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Defining the mechanisms of breast tumorigenesis in the context of chromosome instability and oncogene dependence

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state that: Martina Mantovan made under their supervision the work for the PhD Thesis

"Defining the mechanisms of breast tumorigenesis in the context of chromosome instability and oncogene dependence"

Revising the present work, they state that it encompasses the necessary merits to present and defend it in the view of acquiring the title of Doctor of Philosophy at the University Autonóma of Madrid.

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Summary

Intra-tumor heterogeneity is a widely demonstrated characteristic of human malignancies (Hanahan and Weinberg, 2011). Despite this, cells that became malignant because of oncogenic driven mutations still rely on their sustained over-expression for survival (Weinstein and Joe, 2006). The concept of oncogene dependence has been the base for modern targeted therapy, which, unfortunately still cannot completely eradicate the disease.
Resistance to targeted therapy might be elicited by mechanisms that cause genomic instability, which is a striking feature of both solid and haemapoietic human tumors (Duijf et al., 2012). The importance of chromosome instability (CIN) is underscored by its association with poor patient outcome in different cancer types, including breast (McGranahan et al., 2012). Furthermore, CIN has been shown to facilitate tumor relapse when found in primary tumors (Sotillo et al., 2010).
CIN can be elicited by several molecular mechanisms (Holland and Cleveland, 2012). Among those, over-activation of the mitotic checkpoint - in particular through the up-regulation of the Mad2 protein - has been frequently observed in a variety of human tumors, including breast (Rhodes et al., 2007). Characterization of the molecular mechanisms responsible for tumor relapse has become a major focus in cancer, and the relevance of CIN in patient's prognosis and survival suggests that the CIN status could be exploited in the clinical setting (Holland and Cleveland, 2012; Pfau and Amon, 2012).

The work presented here focuses on the study of breast cancer, which is worldwide one of the leading causes of death in women. Interestingly, CIN scores are prognostic in certain subgroups (Smid et al., 2011); among those, Her2 positive breast tumors, are characterized by extremely poor outcome.
Given the lack of available CIN mouse models of breast tumorigenesis, we generated new mouse models that would allow us to:

- Faithfully model human disease by introducing CIN into established mouse models of breast cancer,
- Study the role of CIN in breast tumor initiation, progression and relapse,
- Understand the molecular mechanisms of cancer relapse in the face of CIN.

The study of these mouse models lead to the following conclusions:

- In the Her2 and c-MYC oncogenic backgrounds, Mad2 over-expression plays a dual opposing role in breast tumorigenesis: tumor suppressing in primary tumor formation while accelerating tumor recurrence,
- Mad2 over-expression promotes tumor heterogeneity,
- Mad2 induced heterogeneity in the primary tumor gives the possibility to choose among different mechanisms of relapse.

Resumen

Una de las principales características del cáncer en humanos es la heterogeneidad intratumoral (Hanahan and Weinberg, 2011). Las células que adquieren características tomorogénicas debido a determinadas mutaciones necesitan mantener la sobreexpresión de oncogenes para su supervivencia (Weinstein and Joe, 2006). La dependencia oncogénica ha sido la base de la terapia dirigida que, desafortunadamente aún no ha sido capaz de erradicar la enfermedad. Esta resistencia a la terapia puede explicarse a través de mecanismos que causan inestabilidad cromosómica (CIN). Dicha CIN adquiere mayor importancia si consideramos su asociación con la mala prognosis de los pacientes. (McGranahan et al., 2012). Se ha demostrado que la CIN presente en tumores primarios es capaz de promover recidivas (Sotillo et al., 2010). Varios mecanismos moleculares pueden dar lugar a CIN (Holland and Cleveland, 2012). Entre ellos, la sobreactivación del punto de control mitótico en particular a través de la sobreexpresión de Mad2- se ha encontrado en una gran variedad de tumores humanos, entre ellos el cáncer de mama (Rhodes et al., 2007). La relevancia de la CIN en la prognosis y supervivencia de los pacientes sugiere que podría ser explotada para el diseño de nuevas terapias en clínica (Holland and Cleveland, 2012; Pfau and Amon, 2012). Este trabajo se centra en el estudio del cáncer de mama. Los niveles de CIN son indicadores de la prognosis de determinados subgrupos (Smid et al., 2011), siendo extremadamente grave en los tumores de mama positivos para Her2. Dada la falta de modelos para estudiar la CIN en tumorogenesis de mama, hemos generado una batería de modelos que nos han permitido:

- Modelar la tumorogenesis humana mediante la introducción de inestabilidad cromosómica en modelos murinos ya establecidos para el estudio del cáncer de mama,
- El estudio del papel que la CIN cumple en la iniciación, progresión y aparición de recidivas tumorales,
- Entender los mecanismos moleculares de las recidivas tumorales en presencia de inestabilidad cromosómica.

El estudio con los modelos animales presentados en este estudio llevaron a las siguientes conclusiones:

- en escenarios de sobreexpresión de los oncogenes Her2 y c-MYC durante la tumorogénesis de mama, la sobreexpresión de Mad2 juega un papel dual: actúa como supresor en la formación de tumores primarios pero actúa como promotor en la aparición de recidivas tumorales,
- La sobreexpresión de Mad2 promueve la heterogeneidad tumoral,
- La heterogeneidad de los tumores primarios debida a la sobreexpresión de Mad2, otorga la posibilidad de elegir entre diversos mecanismos promotores de recidivas tumorales.

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List of Abbreviations

Akt	Ak thymoma (protein)
APC/C	anaphase promoting complex/cyclosome
BC	before Christ
BRCA1	breast cancer 1
BRCA2	breast cancer 2
Bub	budding inhibited by Benzimidazole
Bub1	budding inhibited by Benzimidazole 1
Cdc20	cell dependent cycle 20
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CENP-E	centromere associated protein E
CEP57	centrosomal protein 57
CIN	chromosomal instability
CKI	cyclin-dependent kinase inhibitors
CMV	citomegalovirus
c-MYC	cellular MYC
DAB	3, 3'-diaminobenzine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
EGFR	epidermal growth factor recerptor
EMBL	European Molecular Biology Laboratory
ER	estrogen receptor
ERBB2	erythroblastic leukemia viral oncogene homolog 2
EtOH	ethanol
FBS	fetal bovine serum
FDA	Food and Drug administration
FFPE	formalin fixed paraffin embedded
FISH	fluorescent in situ hybridisation
FW	forward
G1	growth phase 1 (of the cell cycle)
G2	growth phase 2 (of the cell cycle)
GEM	genetically modified mice
H&E	hematoxylin and eosin
HA	hemagluttinin
Hec1	Highly expressed in cancer protein 1
Her1,2,3,4	Human epidermal growth factor 1,2,3,4
Her2+	Her2 positive (breast cancer)
HPR	horseradish peroxidase
IGFR	insulin-like growth factor receptor

IHC	immunohistochemistry
K14	keratin 14
K8	keratin 8
LMD	laser microdissector
M	mitotic phase (of the cell cycle)
mAb	monoclonal antibody
Mad2	mitotic arrest deficient 2
MC	mitotic checkpoint
MEBM	mammary epithelium basal medium
MEFs	mouse mebryonic fribroblasts
M-FISH	multicolor fluorescent in situ hybridisation
MMTV	mouse mammary tumor virus
mQ	milli-Q
MRI	magnetic resonance imaging
MTPAs	microtubule-targeted polymerizing agents
NIH	US National Institutes of Health
NSCLC	non small cell lung cancer
ON	over night
p27	protein 27
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pen/Strep	Penicilline/Streptomycin
PI3K	phosphoinosidite 3 kinase
PKC	protein kinase C
PLC	phospholipase C
PR	progesteron receptor
PTEN	phosphatase and tensin homologue
qPCR	quantitative Polymerase Chain Reaction
Raf	rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolution per minute
RT	room temperature
rtTA	reverse tetracycline trans-activator
RW	reverse
S	synthesis phase (of the cell cycle)
S. cerevisiae	*Saccaromices cerevisiae*
SAC	spindle assembly checkpoint
SCF	Skp, Cullin, F-box (containing complex)
S-CIN	segmental chromosome instability
SEM	standard error of the mean
Skp2	seventeen kilodalton protein 2
Tet	tetracycline
TFAP2C	transcription factor AP-2 gamma
TP53	tumor protein 53
tTA	tetracycline trans-activator
W-CIN	whole chromosome instability

Preface

The origin of the word cancer dates back to Ancient Greece. The Greek physician Hyppocrates (460-370 BC) the "Father of Medicine", was the first using the words *carcinos* and *carcinoma* to define non-ulcer and ulcer forming tumors (American Cancer Society®).
To the best of our knowledge, cancer and human beings have always co-existed and the very first evidence can be found among human mummies and fossilized tumors of the bone tissue. The Edwin Smith surgical papyrus (3000-2500 BC, Ancient Egypt), the oldest known surgical treatise on trauma, is the earliest written prove of cancer description. One of the cases concerning ailments of the soft tissue of the breast describes buldging tumors and was classified with unfavorable prognosis. If the diagnosis reported that "the tumors had spread over the breast, were cool to the touch and buldging" (The University of Chicago, The oriental institute. http://oi.uchicago.edu/), no treatment would help. This appears to have been based on established practice.

How far are we now from the non-treatable disease described more than 5000 years ago?
Since then, research has made immense improvements in understanding the biology and consequent potential tumor treatment. There is an enormous body of literature that supports the hypothesis of cancer developing as a multistep disease, where different somatic mutations must be acquired in order for a cell to become tumorigenic. "Six distinct, but complementary, biological capabilities fuelling transformation have been identified and defined as hallmarks of cancer" (Hanahan and Weinberg, 2000). All of them allow malignant cells to survive in conditions that would inevitably lead to death in normal cells.
Albeit the hallmarks constitute an organizing principle to reconcile the intricacies of the neoplastic disease, the main obstacle is within the term cancer itself. Cancer is not a unique disease, but it encompasses a multitude of heterogeneous tumor types.
Underlying this, are the next generation of cancer hallmarks (Hanahan and Weinberg, 2011): new emerging attributes (evading immune response and altering energy metabolism) and enabling characteristics (inflammation and genome instability).

Since Theodor Boveri (1862-1915) postulated that cancer cells may arise from single cells with abnormal chromosome complement (seminal treatise "*Zur Frage der Entstehung Maligner Tumoren*"), the role of chromosome instability (CIN) in tumorigenesis has been a central question in cancer biology. In fact, genome instability can generate the genetic diversity required to accelerate traditional hallmarks acquisition and, consequently, help the selection of drug resistant clones.

The work presented in this thesis focuses on CIN - a dynamic state in which cells continuously gain or lose whole, or parts of, chromosomes - which is a common type of genome instability, present both in solid and hematopoietic human tumors.
In particular, its role in breast tumorigenesis, from tumor initiation to progression and relapse will be presented and discussed.

Chapter 1

Introduction

1.1 The Mad2 (mitotic arrest deficient 2) protein

1.1.1 The eukaryotes cell cycle

A basic eukaryotic cell cycle consists of four phases: the gap before DNA replication (G_1), the DNA synthetic phase (S), the gap after DNA replication (G_2) and the mitotic phase (M) which culminates in cell division. Since no dramatic morphological changes can be observed, the G_1, S and G_2 phases are collectively known as interphase. Cells in G_1 can, before passing the restriction point and therefore commit to DNA replication (Pardee, 1974), enter a resting state called G_0. In mammals, most of the adult cells are in a quiescent phase named G_0. Active proliferation is a prerogative of specialized cells only, such as certain ones the gut epithelium or the hematopoietic system.
The quiescent phase is a reversible state - with the only exception being terminally differentiated cells. In fact, the presence of specific mitogenic signals allow cells in G_0 to re-enter the cell cycle and resume proliferation and division programs. As soon as the restriction point (Pardee, 1974) in G_1 is reached and passed, cells are committed to replicate their genome and complete mitosis.

Different cellular proteins tightly regulate the transition from one cell phase to another. These are cyclins and their partners CDKs (cyclin-dependent kinases). CDKs are a family of serine/threonine protein kinases that are temporally and specifically activated during the cell cycle. Only five out of nine known CDKs are active during the cell cycle: CDK4, CDK6 and CDK2 during G_1, CDK2 in S, and CDK1 along G_2 and M phases (Figure 1). As CDKs protein levels do not vary during the cell cycle, their activity is controlled by the presence, or absence, of their activating subunits, named cyclins. In contrast, cyclins levels rise and fall during the cell cycle, since protein availability is controlled by ubiquitin-mediated proteasome degradation at the end of the phase they are required for (Figure 1) (Glotzer et al. 1991; Rechsteiner and Rogers, 1996). This way, they can periodically activate partner CDKs (Evans et al. 1983; Pines and Hunter 1991). Specific types of cyclins are required to activate partner defined CDKs to allow progression through the different phases (Figure 1). For this dissertation, it is important to highlight the interaction between cyclin B and CDK1, which regulates the late events of mitosis. Once CDKs are activated, they induce downstream signaling via phosphorylation of target proteins and make the progression through the cycle possible (Morgan, 1995).
Cyclin binding is an essential prerequisite for CDK activation; however, their activity is controlled as well by phosphorylation or dephosphorylation events on conserved threonine and tyrosine residues (Harper et al. 1995; Paulovich and Hartwell, 1995). Furthermore, two distinct classes of CDK inhibitors (CKI) have been discovered: the INK4 family and the

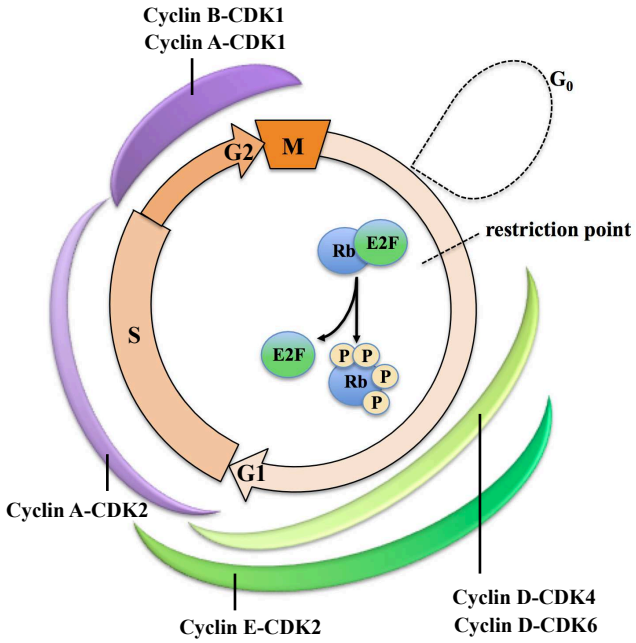

Figure 1.1: Progression through the mammalian cell cycle. The phases of a cell cycle are shown. Cells may enter in quiescence (dotted loop, G_0 phase) during the G_1 phase before reaching the restriction point (dotted line). The progressive activation of cyclin-CDK complexes during the different phases of the cell cycle is also indicated. Re-entry into the cell cycle from quiescence is mediated by cyclin D-CDK4 and cyclin D-CDK6 complexes. Cyclin E-CDK2 complexes are active in late G_1 phase. S phase is characterized by the activation of cyclin A-CDK2. Cyclin A-CDK1 and cyclin B-CDK1 complexes are active during the G_2 and M phases. During G_1, Rb is phosphorylated and inactivated by cyclin D-CDK4, cyclin D-CDK6 and cyclin E-CDK2 complexes. (Figure adapted from (Coller H.A., 2007)).

Cip/Kip family (Sherr and Roberts, 1995). They regulate CDK activity by binding either to CDK alone or to already formed CDK-cyclin complexes. Ultimately, different cell cycle regulating proteins specifically regulate cell cycle progression via their intracellular localization (Heald et al., 1993; Sanchez et al., 1997).

Cyclin protein levels are not only regulated by their degradation. In particular, the E2F family of transcription factors regulate, among other targets, cyclin A and E and CDK1 expression (Ren et al. 2002). In addition, the E2F family is target of the Rb (retinoblastoma) tumor suppressor, the first ever to be identified. Rb activity is regulated by post-translation phosphorylation of conserved residues. When in its active form, Rb binds to E2F and prevents the transcription of target genes that are essential for the G_1 to S transition. However, in early G_1, Rb is phosphorylated by cyclin D/CDK6, cyclin D/CDK4 complexes and, later, cyclin E/CDK2 or cyclin A/CDK2 thus being converted in its inactive form. E2F transcription factors are released and can freely drive the expression of genes essential for later cell cycle phases and DNA duplication. Rb phosphorylation is regulated by extracellular mitogen signals, bridging its tumor suppressive function to cellular proliferation. Thereof, it is of no surprise that the Rb pathway is disrupted in most tumors. Whereas it is mutated only in specific tumor types (i.e. osteosarcoma, retinoblastoma...), increasing evidence has supported deregulation in its activity - through cross-talking signaling pathways - in most of the tumors. Rb deregulated or deficient cells are usually eliminated through p53 mediated apoptosis, since this would allow unscheduled entry into S phase and represent a major danger towards tumor formation. As a consequence, in many tumors both the Rb and p53 pathways are conjointly disrupted to sustain malignant cell growth and uncontrolled division.

As relevant for this dissertation, it is important to mention that E2F transcription factors regulate not only cell cycle genes, but also the expression of the Mad2 (mitotic arrest deficient 2) gene (see 1.2.1). The Mad2 protein is the focus of this work and is an essential component of the SAC (spindle assembly checkpoint, see 1.2.1). Its role, function and consequences of deregulated expression will be described in detail in the next sections. Hernando et al. (Hernando et al., 2004) first demonstrated that Mad2 is, indeed, a direct target of E2F. Consequently - as E2F activity is controlled by Rb - deregulated expression of Mad2 is linked to Rb pathway defects in human patients. Thereof, Rb deficiency not only promotes uncontrolled cell cycle progression, but also genomic instability (see 1.2.2).

1.1.1.1 The mitotic process

Mitosis is the cell cycle phase during which a mother cell duplicates into two genetically identical daughter cells. It was first discovered in the early 1880s by Walter Flemming, who originally coined this term from the Greek word for thread, reflecting the shape of mitotic chromosomes.
Since errors or failure in mitosis generate daughter cells with an abnormal chromosome complement, it is a complex, delicate and highly regulated process.
According to morphological features of the cell, mitosis can be subdivided into five different phases: prophase, prometaphase, metaphase, anaphase and telophase. After the duplicated genome has been correctly distributed and packed into a newly formed nuclear membrane, the mother cell finally divides into two daughter cells in a process known as cytokinesis (from the greek *cyto* - cell - and *kinesis* - motion).

After genome replication in S phase, sister chromatids condensation marks the onset of prophase. Concurrently, the mitotic spindle begins taking shape via the migration of duplicated centrosomes to opposite sides of the mother cell. Prometaphase begins when the nuclear envelope disintegrates and microtubules invade the nuclear space (open mitosis). Sister chromatids are still associated at the centromeric region and multiprotein complexes, called cohesin, help preventing their premature separation (Haering al., 2008). In the meantime, the formation of the mitotic spindle, a symmetrical microtubule-based structure, takes place. In metaphase, the chromosomes have completed congression to the equatorial plate of the spindle, forming the so-called "metaphase plate", and are attached to robust microtubules fibers. In anaphase, the sister chromatids have lost cohesion and segregate towards opposite poles of the spindle. At late anaphase, the spindle elongates separating furthermore the two groups of chromatids. In telophase, chromosomes reach the opposite poles of the spindle, chromatin starts to decondense and the nuclear envelope reforms around the two masses of chromatin.

Finally cytokinesis, the process of cytoplasmatic cell division occurs. It starts during anaphase and progresses through telophase with the assembly of a contractile ring at the cell cortex. This protein filament structure defines the midbody at the equator of the cell and, while shrinking, forms the so-called cleavage furrow. The complete separation of the cytoplasm into the two daughter cells marks the termination of the whole process.

1.2 The cell cycle checkpoints

In 1989, Hartwell and Weinert (Hartwell and Weinert, 1989) postulated the existence of "dependent relationships" during the cell cycle. They hypothesized that the completion of

late events in the cell cycle must have depended on the correct fulfillment of earlier events; only in this case, the correct order of proceedings could be ensured.
"Control mechanisms enforcing dependency in the cell cycle" (Hartwell and Weinert, 1989) were, for the first time, named checkpoints. In this paper, they gave direct evidence for the existence of a DNA damage checkpoint in *S. cerevisiae*. At the same time, they postulated the presence of a dependency of anaphase on metaphase. The control mechanism was hinted by the following observation: whenever a delay in chromosome alignment at the metaphase plate would occur, anaphase was prevented as long as all chromosomes correctly aligned at the plate.

Few years later, two different laboratories (Hoyt al., 1991; Li and Murray, 1991) identified a feedback control preventing exit from mitosis as long as the mitotic spindle was completely formed. Again in *S. cerevisiae*, the use of drugs inhibiting microtubule polymerization led to the discovery of two genes: Mad (Mitotic arrest deficient) and Bub (Budding inhibited by Benzimidazole). These mutant strains were viable as long as mitosis would normally be completed. However, Mad mutants were not able to delay cell division when treated with benomyl and cells were killed only when passing through nuclear division. This suggested that the absence of a functional mitotic spindle was the lethal event when attempting to segregate chromosomes. These studies proved the existence of a Spindle Assembly Checkpoint (SAC) or Mitotic Checkpoint (MC) in budding yeast. We are now aware that its function is to prevent metaphase to anaphase transition as long as all chromosomes are correctly aligned at the metaphase plate and all kinetochores have made connections with the microtubules of the spindle apparatus. Consequently, the SAC ensures accurate chromosome segregation. Given its function, in most metazoans the SAC is a constitutive essential pathway - conserved from yeast to mammals (Benezra and Li, 1996) - whose abrogation causes genomic instability as well as cell and organismal death if happening at early developmental stages. However, while the SAC is needless in organisms such as *S. cerevisiae* or *D. melanogaster* under conditions in which the normal completion of mitosis is not undermined, it is essential in all normal or transformed mammalian cells at every cell division. Hence, genomic abnormalities in human tumors are not likely to be caused by complete SAC abrogation (Schvartzman et al., 2010).

1.2.1 The mitotic checkpoint

Since the Mad2 protein and the mitotic checkpoint are the main focus of this work, the aim of this section will be to give an outline and discuss its main players.

The function of the mitotic checkpoint is to monitor chromosome segregation by delaying anaphase until all chromosomes have become bipolarly attached to the mitotic spindle. Key proteins, essential for mitotic checkpoint signaling, are: Mad1, Mad2, Bub3, and the kinases Bub1 and BubR1. During the early mitotic phases, all of them are at least temporarily localized to unattached kinetochores. The principal target of the SAC machinery is the anaphase promoting complex/cyclosome (APC/C), an SCF E3 ubiquitin ligase whose activity is required for the metaphase to anaphase transition. The MC functions as an inhibitory signal that delays APC/C activation.

In order for mitosis to be completed, two proteins must be eliminated: cyclin B (see paragraph 1.1) and securin. These are both APC targets and undergo ubiquitin-mediated proteasomal degradation; the F-box subunit regulating target specificity is, in this case, the Cdc20 protein (Wang et al., 2013). Cyclin B is the activating partner of the mitotic kinase CDK1,

which is required to enter and maintain mitosis. CDK1 activity decreases following cyclin B degradation, and this event allows transition to late mitotic phases and their fulfillment.
At this phase of mitosis, sister chromatids are still connected at the kinetochores through cohesins, multiprotein ring complexes which prevent their premature separation. Consequently, their elimination is essential to allow chromosome segregation. The protein separase specifically targets cohesins, upon removal of its stochiometric inhibitor securin. Therefore, the SAC prevents both mitotic exit pathways activation - by stabilizing cyclin B - and premature loss of sister-chromatids cohesion prior to correct attachment to the mitotic spindle keeping the protease separase in its inactive state.

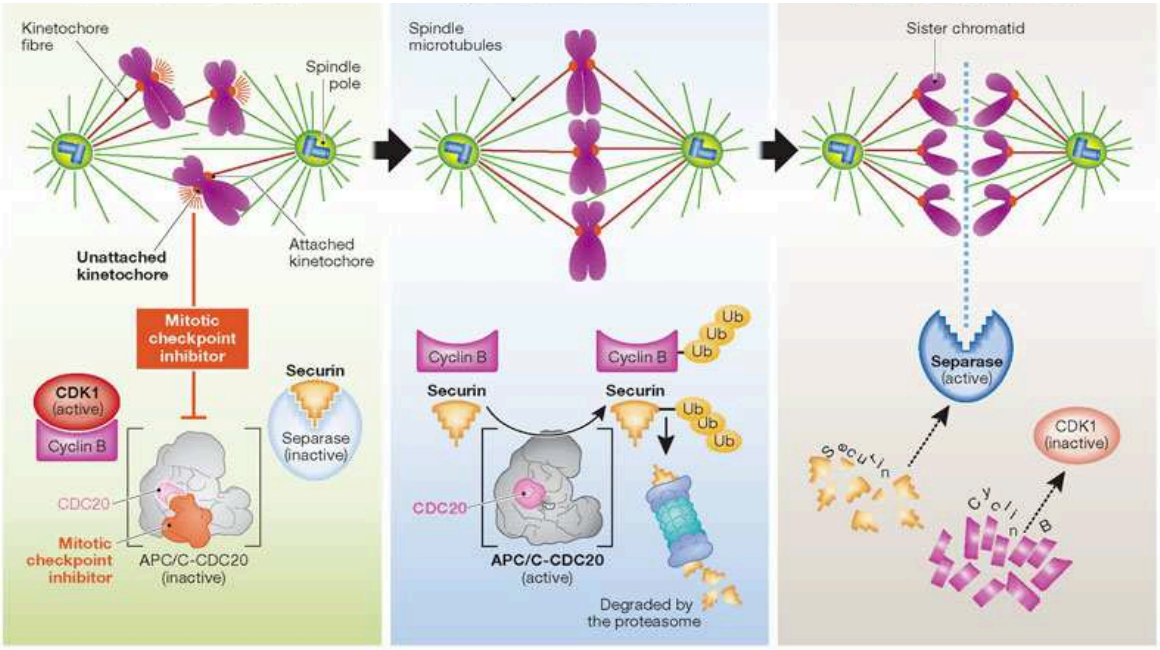

Figure 1.2: The mitotic checkpoint: the guardian of accurate chromosome segregation. Left panel: to prevent chromosome missegregation and aneuploidy, cells have evolved a surveillance pathway known as the mitotic checkpoint that prevents metaphase to anaphase transition until all chromosomes have bipolarly attached to the mitotic spindle. Unattached kinetochores release a diffusible signal that inhibits ubiquitination of cyclin B and securin by the APC/Cdc20 ligase. Middle panel: at metaphase, when all kinetochores are correctly attached, the mitotic checkpoint is silenced and APC/Cdc20 ubiquitinates securin and cyclin B1, thereby targeting them for proteasome degradation. Right panel: destruction of securin frees separase, which promotes sister chromatid separation, and cyclin B1 degradation inactivates CDK1, thus promoting exit from mitosis. (Figure taken from (Holland and Cleveland, 2012)).

Trying to rule out how the SAC machinery works has been a great challenge since the past decade, and, still, we do not have full understanding of it.
It has been widely demonstrated that the sensor of the SAC is the Mad2 protein. It is recruited to the kinetochores by Mad1, via interaction with its N-terminal domain. The Mad2/Mad1 complex is a stochiometric tetramer which disappears from kinetochores upon microtubule attachment. The first model that tried to explain how the SAC works was proposed by Luo et al. (Luo et al., 2004) and was defined as the "exchange model". One year later, the Mad1/Mad2 complex was proposed to act as a primer for Mad2 activation (De Antoni et al., 2005). In fact, the Mad2 protein adopts two conformations: open Mad2 (O-Mad2) and closed Mad2 (C-Mad2), differing for a structural change in the C-terminal segment. Only when in complex with Cdc20 or Mad1, Mad2 adopts the closed conformation. As conformational changes require energy expenditure, kinetochore localized Mad1 was hypothesized to favor the transition from O-Mad2 to C-Mad2 and, at the same time, act as

a receptor for O-Mad2. When converted into the closed conformation, Mad2 is shuffled in complex with the Cdc20 F-box protein. In this model, Mad1/C-Mad2 acts as a template to generate a structural equivalent Cdc20/C-Mad2 copy and, for the first time, provides a mechanism for amplification of the SAC signal in distance from the kinetochores.

Further implementation of this model came a few years later (Kulukian et al., 2009). Direct evidence that unattached kinetochores can catalytically generate a diffusible Cdc20 inhibitor or inhibit Cdc20 already bound to APC/C was given. In this case, unattached kinetochores act directly on Mad2 to amplify the "wait signal" and Mad2 dimerization is required for kinetochore amplification of Cdc20 inhibition. Thereof, C-Mad2/Cdc20 represents a precursor to promote exchange of Cdc20 onto BubR1. Next, Bub3 binding generates the Cdc20/BubR1/Bub3 complex which prevents cyclin B ubiquitination both when Cdc20 is bound to the APC/C and also precluding its binding to the E3 enzyme. As Mad2 was never co-purified with the Cdc20/BubR1/Bub3 complex, its presence might not be constitutive but may act as a shuffling component.

At the same time, the SAC is regulated by the inhibitory activity of $p31^{comet}$ (Yang et al., 2007). In particular, this protein has a structural fold that closely mimics that of C-Mad2 and is, therefore, able to compete for the binding at the dimerization interface of Mad2. This way, it antagonizes the Mad1-assisted structural modification of Mad2 and promotes the disruption of C-Mad2/Cdc20 complexes. $p31^{comet}$ role controls checkpoint activation and gives a potential explanation for rapid checkpoint inactivation once all SAC conditions are satisfied.

The nature of the exact signal sensed by the SAC machinery is still under investigation. On one hand, the proof of SAC proteins recruitment to unattached kinechores perfectly fits with the hypothesis of SAC activation by unattached kinetochores themselves (Rieder et al., 1994; Li and Nicklas 1995). On the other, what is still a mystery is the ability to distinguish between correct and incorrect attachments, such as synthelic ones. In this case, kinetochores are attached to a correct number of microtubules but emanating from the same spindle pole - instead of opposite ones. Shall we consider microtubules as "pulling ropes", they should not only provide an "all or nothing" attachment signal, but generate a certain tension between sister kinetochores when chromatids start segregating towards opposite spindle poles. If the degree of tension is not correct, the SAC may be able to discriminate among correct and incorrect microtubule-kinetochore attachments. Whether the SAC is hold active by an activating signal - in case of incorrect attachments - or shut down in the presence of bipolar attachments is still not clear. At the same time, we still do not know if these two scenarios may coexist or be mutually exclusive. Recent work on budding yeast (Wang et al., 2014) favors a two step model. In the first place, kinetochore attachment may de-sensitize the SAC for activation (for example, by compromising Mad2 binding to kinetochores). After bipolar attachments, tension on sister kinetochores may definitely silence the SAC and allow progression into late mitosis. It is still unclear whether the proposed SAC silencing network is conserved and which proteins could represent the respective counterpart in higher eukaryotes. More work will be needed to elucidate these aspects and merge them with the previous findings.

1.2.2 Aneuploidy as a consequence of a deregulated mitotic checkpoint

The direct progeny of checkpoint deficient cells is usually viable, unless the formation of the mitotic spindle is perturbed (such as in the presence of spindle poisons). In fact, chromosome

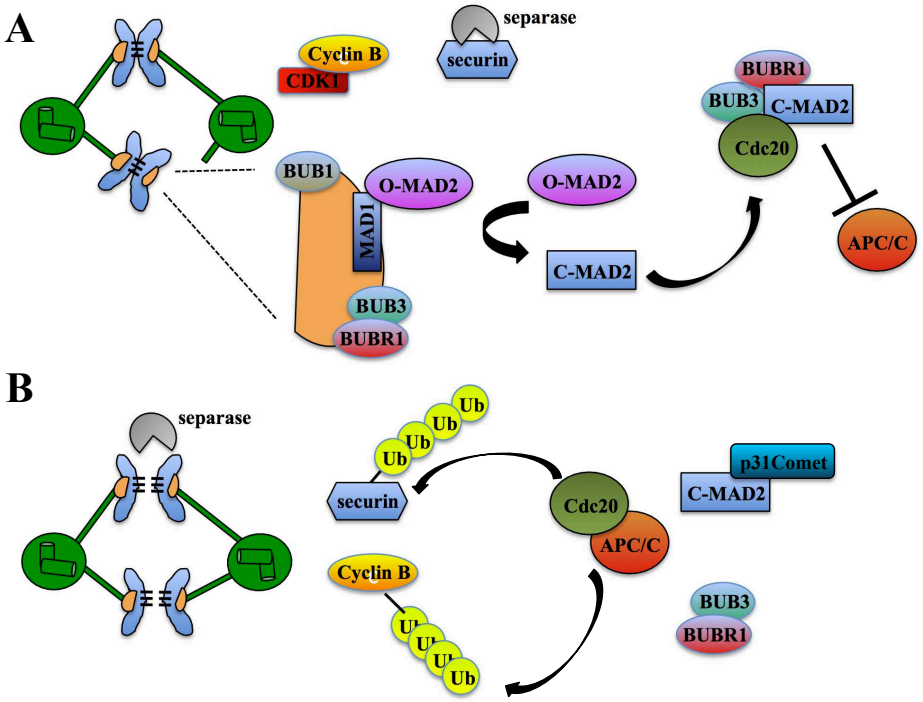

Figure 1.3: Outline of the molecular players of the mitotic checkpoint. A) As long as the last kinetochore is not properly attached to the mitotic spindle, the SAC is active and the Bub3/BubR1/Mad2 prevents Cdc20 binding to the APC/C complex, thus protecting cyclin B and securin from degradation. B) When all kinetochores are attached to microtubules coming from opposite spindle poles, the checkpoint is satisfied and APC/Cdc20 can target securin and cyclin B for ubiquitin (Ub) mediated degradation. Separase can cleave cohesin protein complexes so that chromatids are free to segregate towards opposite spindle poles. This allows metaphase to anaphase transition and further progression of the cell cycle.(Figure adapted from (Geert et al., 2005)).

segregation may still take place even beyond perfection. Nevertheless, MC deficiency will inexorably lead to increased aneuploidy and cell death over time.

If the SAC is chronically activated, instead, cells are blocked in mitosis for a range of time that varies across different species and different cell types within the same organism. However, cells are unable to keep the mitotic state for indefinite time and, eventually, will complete mitosis through molecular mechanisms that have not been fully understood yet. Cells may adapt and exit mitosis through a mechanism known as "mitotic slippage" (Rieder and Maiato, 2004), which seems to be a recurrent modality in different species. The key event is cyclin B degradation, which surprisingly happens even though the SAC has not been satisfied. Upon mitosis completion, cells are invariably aneuploid (Brito and Rieder, 2006). What happens to this immediate progeny will depend on the degree of aneuploidy, the amount of DNA damage that has been generated and on the cell type itself. For instance, if the p53 pathway is active, the outcome may be represented by G_1 arrest followed by senescence or apoptosis. In contrast, cells lacking p53 could survive and further divide, generating even more aneuploid daughter cells.

Cells facing a prolonged mitotic arrest may also be directed to a cell death path known as "mitotic catastrophe" (Vakifahmetoglu et al., 2008). "This term is used to describe an atypical mechanism of death that senses mitotic failure and responds to it by driving the cell into an irreversible state, be it apoptosis, necrosis or senescence" (Vitale et al., 2011). It has

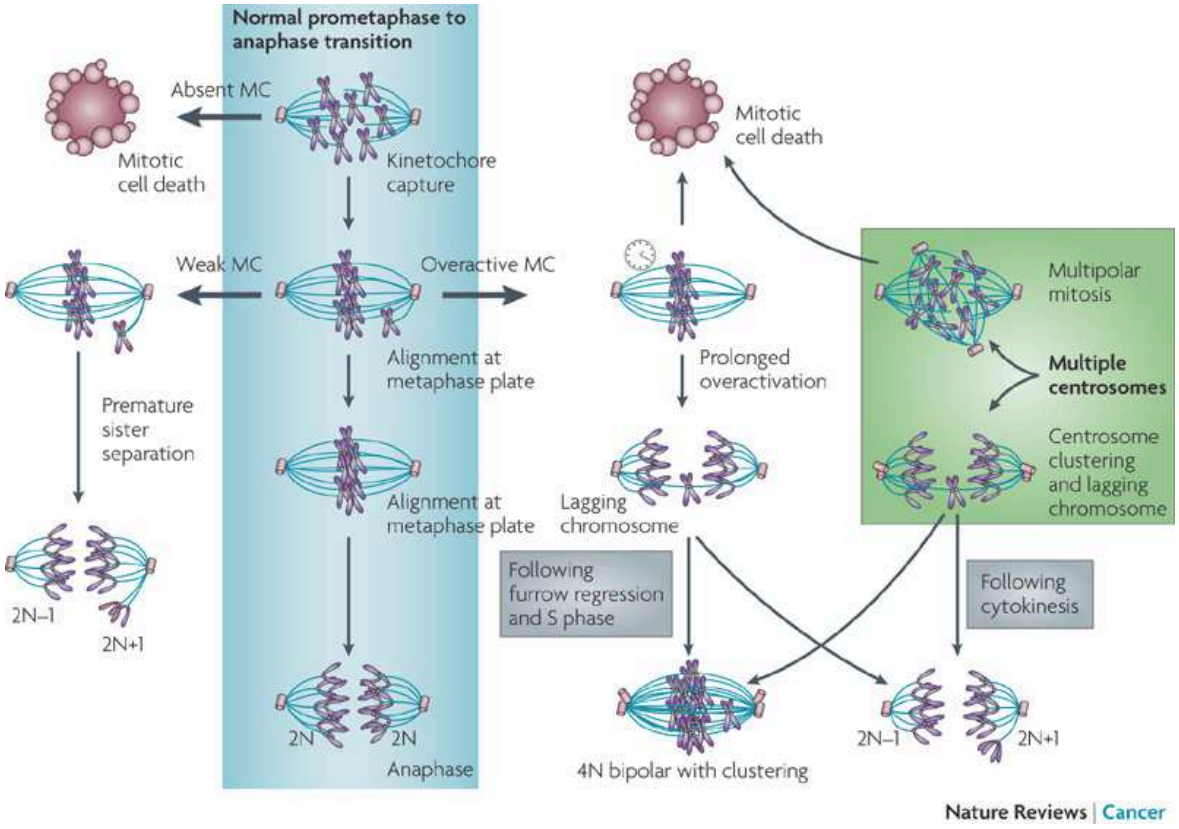

Figure 1.4: Mechanisms leading to aneuploidy. The normal SAC events are shown in the center and highlighted in blue. On the left: loss of the MC leads to cell death. Down-regulated MC favors premature separation of sister chromatids and near-diploid aneuploidy. On the right: consequences of an over-active checkpoint may be either cell death or lagging chromosomes which, thereafter, give rise to near diploid or tetraploid cells. Outcomes similar to an over-active chekcpoint - such as cell death or lagging chromosomes - are generated by multiple centrosomes. (Figure taken from (Schvartzman et al., 2010)).

been related to various morphological and biochemical changes; however, the precise molecular players that regulate mitotic catastrophe have not been characterized yet. Incorrect chromosome distribution in mitosis may compromise cellular function, reduce cellular fitness or, most importantly contribute to tumorigenesis. For these reasons, mitosis-incompetent cells should be eliminated and mitotic catastrophe could be seen as an onco-suppressive mechanism whose aim is to protect against genomic instability (Vitale et al., 2011).

1.3 Aneuploidy and CIN in human tumors

In this part, the relationship between aneuploidy and human tumors will be developed with particular emphasis to deregulation of the mitotic checkpoint as CIN trigger. It is important to mention that alteration of the SAC is not the only mechanism that can generate aneuploidy *in vivo*. However, this has been reviewed elsewhere (Holland and Cleveland, 2012) and is not the aim of this work.

Even normal cells can occasionally missegregate chromosomes in the presence of a fully functional mitotic checkpoint (Hartwell and Smith, 1985). This generates aneuploidy, but the consequences at organismal level are, usually, not harmful and cells with unbalanced chromosome number are strictly controlled. In contrast, in human tumors many cells ac-

quire CIN, a condition characterized by continuous gain and loss of chromosomes at every mitotic event. A recent study showed that 68% of human solid tumors are aneuploid (Duijf et al., 2012). CIN is the characteristic of many aneuploid cancer cells, and the source of constantly evolving karyotypes and tumor heterogeneity. This phenomenon has been associated with resistance to therapy and poor patient prognosis (Kuukasjarvi et al., 1997; Gao et al., 2007; Choi et al., 2009; McClelland et al., 2009; Heilig et al., 2010).

It is fundamental to highlight that aneuploidy and CIN are not synonyms. In fact, aneuploidy describes a state of non-euploid chromosome content, while CIN defines a rate of karyotypic change. Consequently, while CIN cells can be defined aneuploid as well, this correlation is not bidirectional and some tumors may be stably aneuploid without the acquisition of further karyotypic changes.
As point of nomenclature, W-CIN (whole chromosome instability) is a condition defined by gain or loss of entire chromosomes and results in numerical chromosomal abnormalities. On top of this, in cancer cells rearrangements in chromosome structure, such as translocations, deletions, inversions or amplifications, can also be observed and this condition is known as S-CIN (segmental chromosome instability (Geigl et al., 2008; Ricke et al., 2008)).

1.3.1 Over-activation of the SAC promotes CIN in human tumors

As mentioned in 1.2.2, deregulation of the mitotic checkpoint leads to aneuploidy. Theoretically, mitotic errors can be generated both via down regulation or overactivation of the SAC machinery. Nevertheless, it is important to identify the real events that could promote tumor formation *in vivo* and which ones are to be found in human patients.

Since complete abrogation of the mitotic checkpoint is lethal, a weakened SAC is the only status to be considered as CIN source. One could predict that in this condition, the SAC could be satisfied at a lower threshold, thereby generating W-CIN as a consequence of premature separation of sister chromatids. Indeed, this hypothesis has been confirmed in several mouse models bearing conditional or hypomorphic mutations in mitotic checkpoint genes (Kalitsis 2000; Michel et al., 2001; Babu et al., 2003). High aneuploidy is a characteristic of both mouse embryonic fibroblasts and tissues; several of these models (i.e. Bub1 hypomorphic mice and Mad1, Mad2 and Cenp-E heterozygous mice) show an increase in tumor susceptibility.

However, these results have found direct evidence in humans only in sporadic cases. As a matter of fact, extensive analysis of human aneuploid tumors (Perez de Castro et al., 2007) have pointed out that mitotic genes are rarely mutated in human cancers. Downregulation of the SAC is also extremely rare.
So far, mosaic variegated aneuploidy is the only genetic disorder associated with mitotic checkpoint functions and compatible with complete embryonic development, that has been discovered. Mosaic variegated aneuploidy (MVA; OMIM 257300) is a rare autosomal recessive disorder, caused by germline mutations in the checkpoint protein BubR1 or the centrosomal protein CEP57 (Snape et al., 2011). MVA patients have severe aneuploidy and therefore, this proves that defects in the SAC machinery can cause aneuploidy in humans. These patients are characterized by an extremely severe phenotype and the absence of other related syndromes reinforces the hypothesis that loss/inactivation of the mitotic checkpoint is not an event likely to happen in humans. This underlines, again, the concept that the MC is an essential pathway in mammals.

The most convincing evidence against mitotic gene downregulation in human cancers is given by the expression profile analysis of aneuploid tumors (Rhodes et al., 2004; Rhodes et al., 2007). Carter et al. (Carter et al., 2006) extrapolated a chromosome instability signature (CIN 70 from the 70 highest scoring genes) from expression profiles of genes that were consistently correlated with total aneuploidy in six different cancer types. In particular, the CIN 70 signature predicted and stratified breast cancer patients with histological grade 1 and grade 2 lesions according to clinical outcome. Cells with high aneuploidy levels and the CIN 70 signature may correlate for three reasons. First, cells with aneuploid DNA complement may need to synthesize higher levels of proteins involved in the mitotic checkpoint as well as in DNA duplication. Second, if the SAC machinery was impaired (a condition which has been discussed as scarcely possible in this chapter), aneuploid cells may need to produce more of its components as a natural compensatory mechanism. Third, over-expression of CIN genes may allow completion of mitosis in the presence of functional checkpoints, resulting in higher proliferative advantage and possibility to fuel tumor heterogeneity.
Many of the mitotic genes associated with CIN are periodically expressed during the cell cycle, under the control of the E2F transcription factor (Ren, 2002). As a consequence, also an impaired Rb pathway may alter their expression (Schvartzman et al., 2011). Given this, it could have been possible that the prognostic ability of the CIN 70 signature proposed by Carter et al., was due to the detection of the proliferation rate rather than CIN itself. However, gene sets representing the mitotic spindle assembly and spindle checkpoint highly correlated with the CIN signature and their scores were significantly higher if compared to the cell cycle genes. This is consistent with the hypothesis that the CIN70 signature is indeed a prognostic marker and not a simple reflection of an increased mitotic rate.

As piece of evidence from mouse models, Mad2 and Hec1 conditional overexpression (Diaz-Rodríguez et al., 2008; Sotillo et al., 2007) has been shown to be sufficient for generating aneuploidy and initiating tumor formation. Both models are predicted to generate mitotic arrest and, consequently, W-CIN. Moreover, Mad2 overexpression has been to shown to lead not only to W-CIN but also to double strand breaks, deletions and amplifications (Sotillo et al., 2007). In these mice, continuous Mad2 over-expression was not required for tumor maintenance, suggesting that once a karyotype has been selected, it can be stably maintained in the absence of CIN if it confers an advantage for malignant progression.
As briefly mentioned before, a recent work showed that Mad2 over-expression is the mediator of CIN upon inhibition of the Rb and p53 pathways (Schvartzman et al., 2011), which are the two most commonly deregulated pathways in cancer. Mad2 expression is controlled by the E2F transcription factors and loss of Rb or p53 leads to its over-expression. Thus, over-activation of the SAC machinery may be directly caused by deregulation of one of its components as well as through abnormalities in upstream connected pathways.

Work on human cell lines provides an explanation for Mad2 induced CIN (Kabeche and Compton, 2012). This paper unravels a new role for the Mad2 protein, which is independent of its function in the SAC machinery because it does not rely on Mad1 mediated recruitment to the kinetochores (see 1.2.1). When Mad2 is over-expressed, kinetochore-microtubule attachments are hyperstabilized and, therefore, the possibility of correction of merotelic attachments is strongly impaired.
Among other known regulators of kinetochore-microtubule attachments, Aurora B seems to be affected by Mad2 over-expression. The amount of Aurora B at centromeres is reduced and its functional activity is also affected. In fact, kinetochore-microtubule attachments are destabilized by Aurora B action. Thereby, Mad2 over-expression indirectly acts on Aurora B to attenuate the degree of kinetochore-microtule destabilization. As consequence, the rate

of lagging chromosomes and CIN are greatly increased.

Thus, data from cell lines, mouse models and, most importantly, human samples have provided evidence that over-activation of the SAC is a common phenomenon in human tumors and can be causative of CIN *in vivo*. Therefore, MC over-activation, rather than downregulation, might be the primary cause of aneuploidy and CIN in patients.

1.3.2 The aneuploidy paradox

Since aneuploidy is frequently observed in human tumors, this notion has been used to hypothesize a possible role of CIN in tumorigenesis. However, several lines of evidence have demonstrated that aneuploidy has a clear antiproliferative effect and the debate on its tumor promoting or tumor suppressor function is still open (Holland and Cleveland, 2009).

As already mentioned, aneuploidy is detrimental, or even lethal, during development. In humans, germline aneuploidy is a considerable barrier towards complete embryo formation and, in adults, is the leading cause of mental retardation (Hassold et al., 2007; Brown S., 2008). All human autosomal monosomies are lethal and only autosomal trisomies of chromosomes 13, 18 or 21 are viable. Only trisomy 21 (Down's syndrome, OMIM 190695) is compatible with life until adulthood. Aneuploidies of human sex chromosomes are much better tolerated and, for instance, Klinefelter's syndrome (47, XXY; OMIM 400045) is the most common known aneuploidy after trisomy 21. Development up to puberty is usually normal, however fertility and cognitive capacities may be impaired in adulthood, even though there is high phenotypic variability among patients bearing the same karyotype.

Several groups have focused on the correlation between aneuploidy and cellular growth *in vitro*. These studies proved that, in this context, chromosome imbalances are detrimental and results are consistent both in *S. cerevisiae* and *M. musculus* (see 1.3.3).
Under normal growth conditions, yeast strains with supernumerary chromosomes (Torres et al., 2007; Pavelka et al., 2010) or trisomic MEFs cell lines have impaired growth rates and altered metabolism. At least in *S. cerevisiae*, aneuploidy detrimental effect is not due to the increase of DNA content *per se* (Torres et al., 2007), but it correlates to the number of genes encoded by the exogenous amount of DNA (Torres et al., 2007; Williams et al., 2008). The forced production of unnecessary proteins could cause an inhibitory effect, called "proteotoxic stress", whose ultimate readout is impaired cellular growth *in vitro*. This observation could also explain why aneuploidy leads to higher sensitivity in response to drugs that target protein synthesis, folding and degradation (Tang et al., 2011). However, studies on aneuploid human tumors showed that extra chromosomes do not preferentially have a low gene copy number, suggesting that, at least in long term tumorigenesis, proteotoxic-mediated growth impairment does not constitute a barrier *in vivo* (Duijf et al., 2012). Thus, *in vitro* data do not recapitulated what is observed in human tumors.

Then, if 68% of human solid tumors are aneuploid (Duijf et al., 2012), how does this information correlate with aneuploidy as being detrimental to cellular growth and fitness?

One should consider that *in vitro* experiments are carried out in the absence of environmental selective pressure (i.e. nutrients, oxygen, growth factors etc.), thus, clones are selected for the fastest doubling time. Conversely, tumor cells must acquire the ability of constant growth in changing intracellular and extracellular environments. Therefore, tumors might trade a reduced proliferation rate for an increased capacity to adapt and evolve. Intere-

stingly, a slow proliferation rate in human colorectal cancers has been linked to increased tumor aggressiveness and ability to metastasize (Anjomshoaa et al., 2009), and aneuploidy is linked to poor patient outcome in those cancers (Araujo S.E., 2007).
For instance, aneuploid yeast strains proliferated significantly better, if compared to euploid counterparts, under severe genetic or environmental pressures (Pavelka et al., 2010). Furthermore, the emergence of drug resistance is also promoted by CIN in the presence of selective stress conditions (Chen et al., 2012).

This highlights the fact that aneuploidy has not only one possible outcome. Even if CIN may be detrimental for cellular fitness, its impact seems to depend mostly on the particular karyotype and the environmental conditions. As a matter of fact, somatic aneuploidy seems to be well tolerated in many contexts *in vivo* (Babu et al., 2003; Jeganathan et al., 2007; Michel et al., 2001; Weaver et al., 2007). Tumors must cope with constantly changing environments; therefore selection for specific aneuploid karyotypes could confer an advantage during the tumorigenesis process and represent a worthy trade off in the long run.

1.3.3 Aneuploidy can act both as a tumor suppressive and a tumor promoting event

If aneuploidy consequences are context-dependent, then two different, not mutually exclusive, outcomes are envisaged: tumor suppressive and tumor promoting. Down syndrome patients are one of the most outstanding exemplification of this concept, since they have a reduced rate of solid tumors opposed to a significant increase in hematological malignancies (Satgé et al., 1998; Hasle et al., 2000; Yang et al., 2002).

Mouse models served as confirmation of this context-dependent aneuploidy phenomenon. For instance, aneuploid CENP-E heterozygous mice showed an increase in lung tumors and spontaneous lymphomas, but had decreased incidence of carcinogen-induced tumors and, when crossed with $p19^{ARF}$ knockout mice, had longer tumor-free survival (Weaver et al., 2007). Crossing of haploinsufficient Bub1 mice into different genetic backgrounds had, again, opposite outcomes: when in combination with $p53^{+/-}$ or $APC^{Min/+}$ mice tumorigenesis was favored, but it was suppressed if together with PTEN heterozygosity (Baker et al., 2009). Interestingly, haploinsufficiency of BubR1 within the same model decreased the rate of small intestinal tumors and at the same time promoted colon tumorigenesis in the $APC^{Min/+}$ mouse model (Rao et al., 2005).

One explanation for these observations is that different levels of aneuploidy are uniquely dealt with in specific tissues and environments. Low aneuploid levels may still be compatible with cell viability and, in particular karyotypic combinations, promote tumorigenesis. In contrast higher levels may promote cell death and reduce tumor incidence. This concept has been recently demonstrated from the Weaver lab (Silk et al., 2013). Mice heterozygous for the CENP-E protein were crossed into different genetic backgrounds: $ARF^{-/-}$, $Mad2^{+/-}$ and $INK4a^{-/-}$. Both $ARF^{-/-}$ and $Mad2^{+/-}$ mice were characterized by mild missegregation rates and low CIN. The combined reduction of CENP-E caused higher rates of abnormal mitotic figures, cell death and decreased tumor incidence. In contrast, when in the INK4a null background, CENP-E heterozigosity did not suppress tumor formation. This contrast finding can be explained considering that INK4a tumor controls the G1-S transition of the cell cycle and is not directly involved in aneuploidy prevention. Therefore, CENP-E protects mice from tumor formation only when acting in concert with an already pre-existing mild level of CIN: the induction of higher levels of aneuploidy induce cell death thereby

increasing animal lifespan. This finding is of extreme interest, since increasing chromosome missegregation rates in already unstable aneuploid tumors could be considered as a valuable therapeutic strategy.
Consistent with this view, intermediate levels of CIN have been associated with a poor outcome in ER-negative human breast cancer, whereas high levels of CIN are correlated with improved patient long-term survival (Birkbak et al., 2011; Roylance et al., 2011).

Loss of heterozygosity is another event promoted by CIN that may help malignant transformation. This is of particular relevance in a tumorigenic context if main tumor suppressor genes are represented only by one functional copy. Again proof for this phenomenon comes from *in vivo* studies on mouse models (Baker et al., 2009). BubR1 insufficiency - which enhances chromosome missegregation - promotes loss of heterozigosity of the wildtype allele if crossed in the $p53^{+/-}$ or $APC^{Min/+}$ backgrounds. In the first case, mice are predisposed to the formation of thymic lymphomas, while colon tumors arising in the $APC^{Min/+}$ models, instead, consistently gained the mutant copy of the allele at the expenses of the wildtype one.

As recently reviewed (Janssen and Medema, 2012), mouse models that combine numerical CIN and human relevant oncogenes in breast cancer are still scarce. This, despite evidences of the high incidence of CIN in human tumors and the fact that oncogene dependent mouse models often result in karyotipically stable tumors - a situation that does not reflect the human disease. Recently, three mouse models of numerical CIN have been published, all from the Van Deursen lab (Baker al., 2012; Nam and van Deursen, 2014). In case of BubR1 over-expression, mice were protected against aneuploidy, ageing and had reduced tumor formation even when crossed in a Ras oncogenic background. BubR1 over-expression was hypothesized to increase fidelity of chromosome segregation in mitosis, thereby acting as a protective mechanism against the generation of aneuploid cells. This demonstrates that missegregation errors in mitosis can greatly increase tumor incidence and, if chromosome segregation accuracy is increased, the SAC could be exploited in a preventive manner. The other two models were generated to unravel the role of cyclin B1 and B2 in cancer. Aneuploidy is a characteristic of both transgenic animals and, even though generated through different mechanisms, determines an increase in spontaneous and carcinogen induced tumors.

CIN has been recognized to play a fundamental role in determining the response to anticancer therapies in primary tumors and, after, in tumor relapse. In mice, combined over-expression of Mad2 and $Kras^{G12D}$ promoted primary tumorigenis but did not affect the regression of Kras driven lung carcinomas. However, tumors that experienced Mad2-driven CIN relapsed with a 50% higher frequency after the removal of the Kras oncogene, suggesting that the genetic instability imparted by CIN facilitated the evolution of resistant karyotypes (Sotillo et al., 2010).

These observations support the view that the effect of aneuploidy on tumor development is dependent on, at least, three factors: a specific abnormal karyotype, the particular genetic background and the tissue microenvironment. Of course, we cannot rule out the possibility that more factors could influence aneuploidy outcome. For this reason, CIN mouse models provide an extremely valuable tool to study these variables and, when possible, test the efficacy and resistance to current and potential drugs.

1.4 Human breast cancer and CIN

1.4.1 Breast cancer

Worldwide breast cancer is the second leading cause of cancer-related death in women, topped only by lung cancer. It is estimated to account for 28% of new cancer diagnosis in women. The American Cancer Society estimates that, in 2014, about 295,000 women will be diagnosed with breast cancer and about 40,000 will die of it.
Even though breast cancer treatment and, above all, early diagnosis has majorly advanced since the past decade, this disease is still characterized by unresolved scientific and clinical problems. These are related to prevention, diagnosis, tumor progression, treatment, recurrence and therapeutic resistance.

Trying to shed light onto these aspects is challenging, since breast cancer is not a single disease, but is highly heterogeneous both at the molecular and, consequently, clinical level. Gene expression profiling of tumor sets have revealed five major molecular subtypes of breast cancer: basal-like, luminal, normal breast-like, Her2 positive and triple negative (Her2, ER, PR negative). Molecular differences underline heterogeneous clinical pathologies and some of them can be used to delineate therapeutic approaches.

1.4.1.1 Breast cancer aetiology

Mammary glands are tubulo-alveolar glands, probably derived from modified apocrine sweat glands. 15 to 20 lobes of branched glands constitute the adult mammary gland. The lactiferous duct branches into further smaller ducts, at the end of which alveolar lobules with secretory cells can be found. Epithelial mammary gland cells are lined by a second layer of myoepithelial cells and surrounded by adipose and connective tissue and extracellular matrix. Mammary glands are responsive to hormone signaling (estrogen and progesterone), therefore their activity is regulated by the endocrine system.

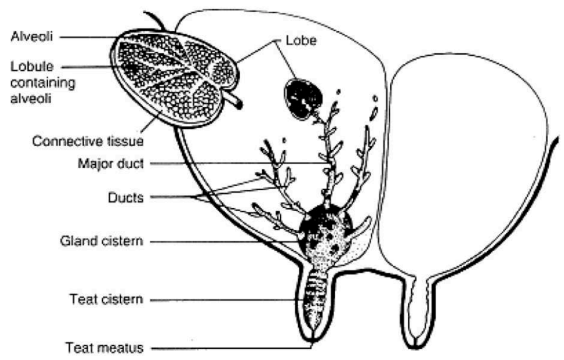

Figure 1.5: Schematic representation of the mammary gland. The basic components of a mature mammary gland are the alveoli (hollow cavities, a few millimeters large) lined with milk-secreting cells and surrounded by myoepithelial cells. These alveoli join to form groups known as lobules. Each lobule has a lactiferous duct that drains into openings in the nipple. Figure taken from http://nongae.gsnu.ac.kr/ cspark/teaching/chap10.html.

As other types of cancers, breast tumors are thought to evolve over long periods of time. Epithelial cells responsible for the transformed state are located in terminal duct lobular units (Welling-Jensen model) and progressively acquire distinct morphological and molecular changes. Evolution of the neoplastic disease starts from hyperplasia, then progresses to atypical hyperplasia, *in situ* carcinoma, invasive carcinomas and, ultimately, metastasis. Increased epithelial growth characterizes initial hyperplasias, and later stages can be identified by loss of cell adhesion and polarity. Further growth and histological heterogeneity marks the entry into *in situ* carcinoma stage, a condition that might be accelerated by genomic

instability. Invasion of the extracellular matrix and surrounding stroma is the prerequisite for spread of the disease to other organs. Breast tumors primarily metastasize to the brain and to the bone, in addition to the lung, usually via the lymphatic system.

At the molecular level, most changes driving breast cancer tumorigenesis fall into two well known categories: gain of function mutations in proto-oncogenes, which are involved in cell growth, division and survival; and loss of function mutations in tumor suppressor genes that normally help prevent uncontrolled cellular growth and promote DNA repair and cell cycle activation (see table 1.1).
Family history and hereditary factors are one of the strongest determinants of risk for breast cancer development. For instance, germline mutations of the BRCA1 and BRCA2 genes account for about 90% of all familial cases and the tumor suppressor p53 is also accounting for a high risk in genealogical trees. Yet, only 5-10% of all newly diagnosed breast carcinomas are linked to genetic factors while the remaining ones are sporadic. Table 1.1 illustrates the most common oncogenes and tumor suppressor genes found in breast cancer. Herein, I will focus specifically on two oncogenes - Her2 and c-MYC - which will be relevant for the work of this dissertation.

Intra-tumor heterogeneity is a widely demonstrated characteristic of human malignancies, which encompasses genetic, epigenetic and karyotypic levels. Despite this, cells that became malignant because of oncogenic driven mutations/over-expression, still rely on the sustained expression of their initial oncogenic lesion for survival, a phenomenon known as oncogene dependence (Weinstein et al., 2008). Supporting evidence has been obtained from cancer cell lines, genetically engineered mouse models and clinical trials involving specific molecular targeted agents. In fact, the concept of oncogene dependence has provided the rationale for modern targeted therapy, whose aim is to provide efficient and safe cancer treatment. However, current therapeutic treatments are only partially successful and cannot completely eradicate the disease. Cancer cells may display primary resistance, if intrinsic factors are limiting drug effect, or secondary resistance if tumors adapt over time to the main drug target or over-ride its efficacy through different pathways. From the clinical point of view, this means that patients are faced, over time, with relapses that do not longer respond to the initial treatment.

- **The c-MYC oncogene**

The c-MYC oncogene encodes a transcription factor with pleiotropic functions, involved in cellular proliferation, differentiation and apoptosis. It is over-expressed or amplified in a relevant part of human breast tumors (15-25%, (Nass S.J., 1997)) and can be associated with more aggressive clinical outcome and worse prognosis. De-regulation in c-MYC expression determinates excessive activation of its downstream pathways, thus leading fertile soil for tumor formation. It is still not clear whether aberrant c-MYC expression alone is sufficient to cause breast tumorigenesis in humans (albeit being proved in mice (D'Cruz et al., 2001)), however its over-expression is clearly associated with breast cancer (Nass S.J., 1997). Interestingly enough for the topic of this dissertation, DNA damage and karyotypic abnormalities may be associated with aberrant c-MYC expression. One critical function of this oncogene, which greatly facilitates the emergence of genome instability, is apoptosis activation. If this response is active, then karyotypically abnormal cells will, most likely, be eliminated. In contrast, if this pathway is not functional, genome destabiliazation can be generated at different levels. For instance, c-MYC deregulation increases metabolic activity and, consequently ROS production. This may generate DNA damage through oxidation and potentially induce rearrangements if DNA breaks are not repaired (Vafa et al., 2002).

Oncogenes	Incidence	Cellular function
Her2	Amplification in 20-30%, somatic mutations 1.6%	Cell survival, proliferation and growth
c-MYC	Amplification and over-expression 15-25%	Cellular proliferation, differentiation, apoptosis
Cyclin D1	Amplification 10-20%, over-expression 40-50%	Cell cycle progression G1-S phase
Cyclin E	Amplification 2%, over-expression 20-30%	Cell cycle progression G1-S phase
Tumor suppressors		
p53	Mutation 20-30%	Cell division, DNA repair, apoptosis
p27 and Skp2	Mutation 1%	Negative regulators of the cell cycle
BRCA1	Mutation 90% familial cases	DNA repair pathway

Table 1.1: Oncogenes, tumor suppressor genes and relative incidence in human breast cancer.

c-MYC also plays a role in the regulation of G_1/S and G_2/M cell cycle checkpoints, and deregulated expression can lead to aberrant DNA replication, which, in turn, causes genomic instability (Santoni-Rugiu et al., 2000; Spruck et al., 1999). Because of the central role of c-MYC in many cellular functions and in every cell, this oncogene is currently not druggable in the clinics and targeted therapy against it does not exist.

• The Her2 (human epidermal receptor 2) oncogene

The Her2 protein is encoded by the ErbB2 gene and belongs to the EGFR (epidermal growth factor receptor) tyrosine/kinase transmembrane receptor family. Four functionally and structurally related members are part of it: EGFR (alternatively named Her1, ErbB1), Her2 (or c-ErbB2; in rodents, Neu), Her3 (alias ErbB3) and Her4 (ErbB4). All of them share a conserved extracellular, ligand binding domain, a transmembrane portion and a cytosolic ATP-binding kinase domain. Extracellular ligand binding leads to receptor activation and conformational changes. Activation relies on dimerization with another protein family member (heterodimerization) or with the same partner (homodimerization). Her2 is known as the "deaf" member of the family, since the lack of ligand domain makes its activation strictly dependent on heterodimerization. Homodimerization of Her2 with itself does happen, however only when expressed at very high levels or in the presence of mutations in the transmembrane domain. Receptor activation results in trans-phosphorylation of their intracellular domain and the possibility of interacting with cytosolic signaling molecules and other membrane signaling pathways (Montemurro and Scaltriti, 2014). These are the Ras/Raf mitogen activated protein kinase, the PI3K/Akt and the PLC/PKC pathways.
In the family, the "mute" Her3 member lacks tyrosine kinase activity; therefore, as Her2, it must heterodimerize with other partners. Even though all heterodimer combinations are possible, the Her2/Her3 one is considered the most powerful in terms of oncogenic potential (Pinkas-Kramarski et al., 1996; Alimandi M, 1995).

As a proto-oncogene, Her2 is expressed at low physiological levels on the surface of epi-

thelial cells and is essential for the correct development of the mammary gland (Browne B.C., 2009). Interestingly, benign breast disorders are rarely characterized by Her2 over-expression (Allred et al., 1992; Mansour E.G., 1994), which usually takes place at later stages. However, 20-30% of malignant breast tumors are characterized by amplification or over-expression of Her2, delineating a specific subtype of disorders defined as Her2 positive (Slamon et al., 1987). Historically, Her2+ breast tumors have had one of the worst prognosis, which correlated with low disease-free and overall survival rates (Paik et al., 2000) compared to Her2 negative ones (Slamon et al., 1987; Seshadri et al., 1993). This is why there has been intense research towards the development of potential targeted therapy drugs against over-expressed Her2 receptor (Yarden and Pines, 2012). First convincing results came from animal studies performed by Greene and Weinberg groups. Fibroblasts ectopically expressing an oncogenic form of rat Her2 acquired the capacity of growing tumors in host animals. This condition was completely eliminated if a Her2 targeted antibody was administered (Drebin et al., 1985). Shortly after (Hudziak et al., 1989), Genetech developed another murine antibody, 4D5, which specifically inhibited the growth of Her2+ cell lines derived from human breast tumors. 4D5 was the predecessor of trastuzumab (Carter et al., 1992; Vu and Claret, 2012), the monoclonal antibody approved in 1998 by the FDA (Food and Drug administration) for the treatment of Her2-metastatic breast cancer. Later, in 2006, it received the second approval for adjuvant treatment of Her2 positive breast cancer. Trastuzumab is one of the most important innovation in the management of breast cancer seen in the last decades, as it reverted the natural outcome of Her2 positive tumors translating the concept of aggressive subtype to the one of improved prognosis. As a matter of fact, cost-effectiveness of trastuzumab regimens has been demonstrated both in combination with adjuvant therapies and in the metastatic settings (Garrison et al., 2007; Liberato et al., 2007; Lidgren et al., 2008). Women with Her2+ breast cancer, treated with trastuzumab, have 44% reduction in risk of death compared to women with Her2 negative disease in primary line treatment. Moreover, by 5 years relapse follow up, survival outcomes are similar in Her2 positive, trastuzumab treated, and Her2 negative patients (Liberato et al., 2007).

Nevertheless, due to either intrinsic or acquired mechanisms of resistance, the median duration of response is modest (Gajria and Chandarlapaty, 2011; Zhang et al., 2011). Unfortunately, less than 35% of Her2+ patients initially respond to trastuzumab, which means that these patients are inherently resistant to the drug. On the other hand, around 70% of patients who initially responded experience progression to metastatic disease within a year (Gajria and Chandarlapaty, 2011), proving the emergence of acquired resistance. So far, three main mechanisms of acquired resistance have been discovered: structural mutations of the Her2 receptor (Christianson et al., 1998), over-expression of other EGFR family members (Garner et al., 2013) and deregulation of expression of downstream components of the Her2 pathway (Berns et al., 2007).

In order to identify gene expression signatures specific for resistance and predict therapy ineffectiveness, profiling of Her2+, trastuzumab treated specimen has extensively been conducted (Khoury et al., 2010; Shiu et al., 2014). These screenings have pointed out the existence of gene sets differentially expressed in Her2+, trastuzumab treated patients versus Her2+ patients that did not receive trastuzumab. In particular, only a fraction of trastuzumab resistant patients remains truly addicted to the ErbB2 oncogene, developing further dependencies (such as the one for the TFAP2C transcription factor) that might be exploited in the clinical setting (Shiu et al., 2014).
One of the reasons why we still heavily rely on gene expression profiling is that we still do not know the exact mechanism of action of trastuzumab and, therefore, the reason for

emergence of resistance to it. To overcome trastuzumab resistance in patients, new drugs against trastuzumab resistant cancers have been developed (Mohamed et al., 2013), leading to alternative strategies in the management of this disease. Even though new drugs can help improving patients overall survival, there is an extreme need to understand the molecular mechanisms underlying acquired resistance to therapy, so that new avenues could be opened in the treatment of the disease.

1.4.2 CIN in breast cancer

Breast tumors appear to be characterized by genome instability even at early disease stages, when tissues still look normal from a histological point of view. As previously mentioned, inherited breast cancer germline mutations - for instance, in BRCA genes - are involved in DNA repair and are, therefore, directly connected to the maintenance of genome integrity. Genome instability, whether inherited or not, results in a greater potential to select for genetic changes (such as gene loss, gene amplification, point mutations and chromosomal aberrations) that would confer an advantage in proliferation, cell survival and drug resistance. Interestingly, loss of heterozygosity and changes in gene copy number seem to be correlated with the transition between hyperplasia and ductal carcinoma *in situ*, thus favoring higher grades of the disease.

In breast cancer, CIN scores are significantly associated with prognosis (Smid et al., 2010) - measured by the time to distant metastasis - and progression in ER+, luminal B and Her2+ subtypes. In contrast, within ER- samples, CIN is likely related to the onset but other factors appear to influence the progression of the disease. As previously mentioned, Carter et al. (Carter et al., 2006), were able to identify an expression signature of 70 genes correlating with CIN that predicts poor prognosis in different types of cancers, including breast. Further refinements of this gene list (Szasz et al., 2013) have proven useful to stratify breast cancer outcome in a specific subset of specimen, further reinforcing the importance of CIN in poor prognosis. In fact, CIN was demonstrated to be an independent variable associated with improved long term survival in ER- breast cancer patients, while it appears to be associated with higher risk of death in ER+ patients (Birkbak et al., 2011; Roylance et al., 2011).

As reviewed by Perez de Castro et al. (Perez de Castro et al., 2007) alterations of genes involved in spindle formation or chromosome segregation are frequent in different types of cancers and represent a direct cause for chromosome instability. These genes are rarely found mutated in human tumors, but one should not forget that also minor expression changes, still compatible with cell viability, might be sufficient to deregulate the mitotic checkpoint and induce genome instability.
Regarding Mad2, only rare mutations have been found in bladder, lung and breast cancer (Gemma et al., 2001; Perez de Castro et al., 2007). As reported in the ONCOMINE database (`http://www.oncomine.org`) (Rhodes et al., 2004), Mad2 is significantly up-regulated in a subset of human cancers, including breast. Further assessment of Mad2 expression from patient specimen has reinforced this notion and linked Mad2 to invasive ductal breast carcinomas (Scintu et al., 2007; Borowsky, 2011).

Mad2 has been long known as an E2F target gene (Hernando et al., 2004) and also deregulation in the p53 axis may alter Mad2 expression. In fact, the p21 kinase is a direct target of p53, and its role is to maintain an hyper-phosphorylated Rb protein preventing unscheduled activation of the E2F transcription factors (Polager and Ginsberg, 2009). Therefore, p53 is also involved in controlling the correct cell cycle timing via indirect regulation of the Rb pro-

tein. This is of tremendous relevance, since deregulation in either Rb/E2F or p53 pathways may lead to Mad2 deregulation, thus fueling CIN and tumor heterogeneity. As a matter of fact, recently published results clearly show that Mad2 over-expression is a consequence of impaired Rb or p53 mutant background and, in this context, represents the principal mediator of CIN (Schvartzman et al., 2011).

Therefore, in order to mimic the unstable aneuploidy seen in human tumors, we decided to use a mouse model that conditionally over-expresses the Mad2 protein (Sotillo et al., 2007). In this way, we can induce genomic instability and study tumorigenesis in an improved mouse model of breast cancer.

1.4.3 Modeling of the human disease

As for other types of cancer, it is not possible to generate a perfect model, since tumors - and breast malignancies as well - are constituted by an array of different diseases. The first and simplest surrogate to study breast cancer is represented by cell culture lines. However, the major drawback is the culture condition itself: these cells grow in 2D onto plastic dishes that have nothing in common with the extracellular tumor environment.
Propagation of human tumor derived cell lines into immuno-compromised host mice is, for sure, a great implementation compared to cell line culture. Nevertheless, it is important to consider that, despite the use of a living organism, xenograft techniques do not allow the study of the immune response, as well as the creation of a permissive microenvironment related to pre-malignant stages. This is why genetically modified mouse (GEM) models have played a pivotal role in the study of tumor diseases since the past century (Allred and Medina, 2008; Borowsky, 2011; Cardiff and Kenney, 2011). They have represented a great advantage towards the generation of models that would encompass both biological complexity at organismal level and human related driving mutations in cancer.

As far as breast cancer is concerned, mouse models have had enormous success for several reasons. First of all, mouse mammary gland biology has been studied for many years and it closely resembles the human one in many aspects, starting from development, tissue architecture, to hormonal regulation and function. In second place, a lot of effort was put into the creation and generation of mammary gland specific promoters, such as the mouse mammary tumor virus long terminal repeat (MMTV-LTR) the way acidic protein (WAP) and beta lactoglobin (BLH). All these promoters are able to drive high transgenic expression specifically in mammary epithelial cells, allowing the design of elegant tissue specific experiments. Last, since the mammary gland is a non essential organ - unless lactation is desired - it is amenable to transplantation experiments (Daniel et al., 2014).

Apart from basic biology features regarding mammary gland biology, one relevant question rising in the field was related to the pathology of breast tumors in mice compared to human specimen. Therefore, in 1999 a meeting of human and mouse breast cancer pathologists (NIH Breast Cancer Think Tank and Annapolis Pathology Panel) developed a consensus report comparing the pathology of 39 GEM and human breast cancers (Cardiff et al., 2000). The major conclusion of this meeting was that spontaneous breast carcinomas arising in mice did not resemble any common type of human breast cancer. However, whenever tumorigenesis was driven by specific transgenes, such as MYC, ErbB2 and Ras, tumors were characterized by unique histologies, hardly distinguishable from the human counterpart. Hence, these results were considered as most significant, highlighting the fact that driving oncogenic lesions profoundly affect tumor pathology both in humans and in mice.

In humans, oncogenes usually drive tumor formation thanks to increased expression, which can be due to deregulated expression or copy number variation. Therefore, the use of highly active promoters in mice provides a valid surrogate to mimic this disease at the molecular level. Further improvements were brought by the use of tetracycline inducible promoter sequences (adopting a mammary specific promoter to drive the tet transactivator and a second tet responsive driven element) and the administration of tetracycline/doxycycline as major rate limiter (Gunther et al., 2002). The specificity and temporal control of transgenes expression of this system has proved critical to a large variety of studies. In case of the Her2 and MYC oncogenes, many tetracycline inducible mouse models have already been generated and have been reviewed elsewhere (Borowsky, 2011).

As relevant for this dissertation and given the importance of the Mad2 protein in human cancers, few mouse models have started giving insight into the biology of CIN tumors focusing specifically on this protein. Sotillo et al. (Sotillo et al., 2007) generated a mouse carrying an inducible HA tagged Mad2 cDNA, under the control of the tetracycline system. In MEFs, Mad2 over-expression was sufficient to transiently increase the number of mitotic cells, which would eventually slip through becoming binucleated or mononucleated with abnormal chromosome content. Mad2 over-expression was also causative of chromosome instability *in vitro* and, *in vivo*, able to initiate tumorigenesis in a wide spectrum of tissues. Moreover, unlike oncogenes, continued over-expression of Mad2 was not required for tumor maintenance. These results demonstrated for the first time, that transient Mad2 over-expression and chromosome instability could be an important stimulus in the initiation and progression of different cancer subtypes. Few years later, the first mouse model combining Mad2 over-expression with a lung tumor relevant oncogene ($Kras^{G12D}$) was published (Sotillo et al., 2010). They showed that induction of chromosome instability by over-expression of Mad2 did not affect the regression of $Kras^{G12D}$-driven lung tumors when targeted therapy was mimicked. However, tumors that experience transient Mad2 over-expression recurred at elevated rates, compared to $Kras^{G12D}$ only induced malignancies. Thus, early chromosomal instability may be responsible for tumor relapse after seemingly effective anticancer treatments.

Nevertheless, studies performed *in vivo* are often long and complicated to reveal detailed mechanistic insights of tumor relapse. This is why three-dimensional cell cultures have progressively taken more importance, as they reduce the complexity of *in vivo* experiments while allowing organotypic growth and studies at single cell level, with respect to cellular organization and position within a tumor (Jechlinger et al., 2009).

Trying to shed light onto the mechanisms of targeted therapy resistance is of extreme importance. Characterization of the molecular mechanisms responsible for tumor relapse has become a major focus in the cancer field, and the relevance of CIN in patient outcome suggests that the CIN status could be exploited in the clinical setting. For these reasons, the present work aimed at the study of CIN in the breast tissue using a mouse model that would faithfully mimic human disease.

Cancer is still a devastating disease and continuous effort should be implemented to study and understand its molecular biology.

Aims

Several questions are still open regarding the connections between oncogene driven breast cancer and chromosome instability.

Analysis of human samples has linked CIN either to tumor onset or progression, and there is a strong correlation between aneuploidy, poor patient prognosis and relapse. Among different mechanisms generating CIN in tumors, over-activation of the mitotic checkpoint has been frequently observed. In particular, gene expression profiling of human breast carcinomas has highlighted that Mad2, an essential component of the SAC, is commonly up-regulated.
Despite the strong evidence supporting the role of CIN in human breast tumors, so far, no mouse model able to reconcile this aspect with the acknowledged concept of oncogene dependence has been generated. As consequence, the role of CIN in oncogene driven breast tumorigenesis has not been deeply investigated.

For these reasons, we generated new mouse models of breast cancer, crossing mice over-expressing the Mad2 protein together with oncogenes (Her2 or MYC) known to be prominent in human patients. This way, we were able to:

- Faithfully model human disease by introducing CIN into established mouse models of breast cancer,
- Study the role of CIN in breast tumor initiation, progression and relapse,
- Understand the molecular mechanisms of cancer relapse in the face of CIN.

Chapter 2

Materials and Methods

2.1 Genetically modified mouse models

2.1.1 Transgenic mice

All the transgenic mouse lines used in these studies were previously generated: TetO-Her2 (Moody et al., 2002), TetO-MYC (D'Cruz et al., 2001), TetO-Mad2 (Sotillo et al., 2007), MMTV-rtTA (Gunther et al., 2002). All mice were on a mixed 129/Bl6/FVB background.

2.1.1.1 Animal housing and husbandry

All mouse procedures were approved by the European Molecular Biology Laboratory Monterotondo Ethical Committee (Monterotondo, Italy) and were in accordance with national and European regulations.

Animals were group housed and kept on a 12h light, 12h dark cycle (lights on at 7 a.m.) with constant ambient temperature (21.5±1°C) and humidity (55±8%). Mice were weaned at 3 weeks of age and housed in same sex groups in 3-5 per cage with pellet food and water *ad libitum*. Immuno-compromised mice were housed in pathogen-free areas and monitored for welfare and health.

2.1.1.2 Doxycycline diet

At 9 weeks of age virgin females were switched to a doxycycline-enriched diet. Doxycyline (625 mg/kg) was administered in impregnated food pellets (Harlan). Upon diet switch, mice did not show differences in food intake.

2.1.1.3 Animal monitoring

Animals set on a doxycycline diet were weekly monitored for the emergence of primary tumors, as well as overall condition, including appearance, posture, behavior and physiological responses, food and water intake. A caliber (VMR) was used to measure tumor size.
Experiments were completed before tumor development or tumor associated disease caused death or a significant deterioration in the host.
Humane endpoint stage was determined according to tumor size (20 mm diameter - if the animal had more than one tumor, this size was the maximum allowable size for all tumors combined) and the presence of one or more criteria for euthanasia (interference with a vital physiological function, labored breathing, prolonged dehydratation, muscle atrophy and signs of lethargy and lack of physical activity, tumor ulceration or necrosis for more than

72h). At humane endpoint stage mice were euthanized to collect primary tumor samples. Otherwise, if needed for relapse monitoring studies, they were set back to a normal diet or biopsied (see 2.1.2.3). Tumors were allowed to regress and mice were constantly monitored for tumor recurrence.

2.1.1.4 Animal genotyping

Genotyping was performed by PCR using genomic DNA from tail tissue after 16h digestion (50 mM KCl, 10 mM Tris pH 8.0, 2 mM MgCl2, 0.45% (v/v) NP-40, 0.45% (v/v) Tween 20, 1 mg/ml proteinase K) at 56°C. Proteinase K (Roche) was inactivated following incubation at 85°C for 30 minutes. PCR amplification was used for transgenes detection in somatic DNA. One µl of each sample supernatant was used for PCR reaction. PCR mastermix was as follows: 0.25 pmol/µl FW primer, 0.25 pmol/µl RW primer, 200 µM dNTPs, Taq Polimerase 1 U/20µl, Dream Taq Green Buffer (Thermo Scientific) 1X. See Table 2.1 for complete primers list. PCR conditions were as follows: 95°C for 1', 2x [95°C for 15", 64°C for 15", 72°C for 1'30"], 2x [95°C for 15", 61°C for 15", 72°C for 1'30"], 20x [95°C for 15", 58°C for 15", 72°C for 1'30"], 10x [9°C for 15", 55°C for 15", 72°C for 1'30"], 72°C for 10'. PCR products were run on a 1.5% agarose (Sigma) gel with ethidium bromide (Sigma) and visualized with Molecular Imager® GelDocTM XR+ instrument (Biorad).

Mouse line	FW	RW
TetO-HA-Mad2	CCATCCACGCTGTTTTGACCTC	GGCTTTCTGGGACTTTTCTCTACG
TetO-Her2	GACTCTCTCTCCTGCGAAGAATGG	CCTCACATTGCCAAAAGACGG
TetO-MYC	TAGTGAACCGTCAGATCGCCTG	TTTGATGAAGGTCTCGTCGTCC
MMTV-rtTA	GTGAAGTGGGTCCGCGTACAG	GTACTCGTCAATTCCAAGGGCATCG

Table 2.1: Forward and reverse sequence of primers used for mouse genotyping.

2.1.2 Surgical techniques

Mice were subjected to surgery only if overall condition was judged adequate prior to procedure. All surgeries were performed on isofluorane (Esteve) anesthetized mice. Surgical bench was cleaned and sterilized according to guidelines. Autoclaved, disinfected surgery tools were used. Mice were placed onto a heating pad to prevent decrease of body temperature. Depilatory cream (Veet) was used to shave the area of interest and, afterwards, exposed skin was disinfected. After surgery, skin wounds were closed using suturing aids (Peters surgical) and 3M Vetbondt tissue adhesive (3M Animal Care Products). Subsequent to surgery, mice were housed into a clean cage placed onto a heating pad. After surgical intervention animals were fed with the corresponding diet complemented with DietGel Recovery (Clear H_2O) nutrient. Prior to any further experiment, mice were let to recover at least for one week.

2.1.2.1 Removal of abdominal mammary glands

Virgin, doxycycline naïve females were subjected to surgery at an age of 8 to 9 weeks. A small incision (1 cm length) was practiced in the abdominal area of the skin. Mammary glands were gently detached from muscles and skin, then resected and placed onto a sterile

dish. Disinfection prior to suture was carefully carried out both in internal and external abdominal area.

2.1.2.2 Mammary fat pad injection of abdominal mammary glands

21 days old immune-compromised Rag $1^{-/-}$ mice (Charles River) were used for mammary fat pad injection. Unlargae mammary glands were removed as described in Brill at al. (Brill et al., 2008), and further processed (see Histological and immunohistochemical analysis). Dissociated primary tumor cells were resuspended in 1:1 cells:matrigel (Trevigen Inc.). 10 µl of 1:1 mixture were injected into each cleared fat pad using a 250 µl syringe (Hamilton).

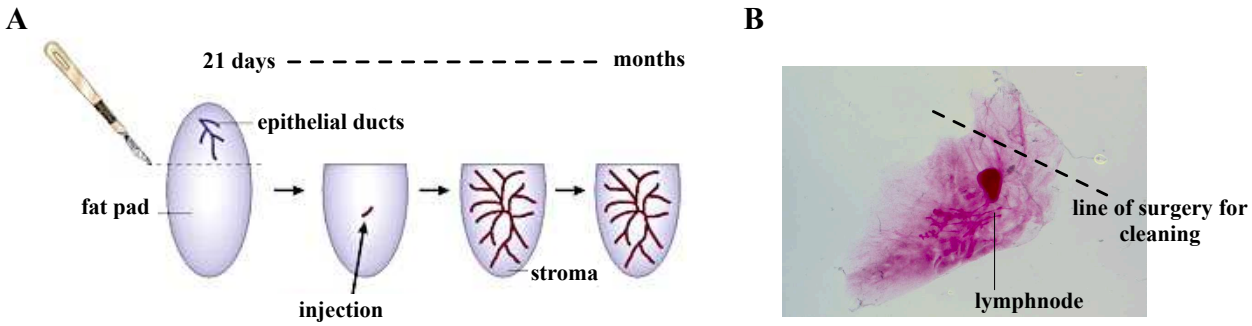

Figure 2.1: Mammary fat pad clearance and injection of immune-compromised Rag $1^{-/-}$ mice. A) Schematic representation mammary gland removal of 21 days old females. The originating center of the mammary gland tree is called unlargae and is resected in order prevent its branching into the fat pad. 1:1 mixture cells:matrigel (Trevigen Inc.) was injected into the fat pad, allowing new cells to colonize and grow into a physiological environment. B) Carnoy staining (see 3.2.1) of cleared mammary gland. The mammary gland unlargae is close to the lymph node, which marks the site of resection for surgery. (Panel A taken from (Brill et al., 2008))

2.1.2.3 Primary tumor biopsy

According to ethical guidelines, a maximum of 2 tumor biopsies (3 mm biopsy punch, Miltex) per mouse was carried out. After disinfection of surgical area, the mouse was shaved (Veet) only along the site of incision. Nude skin was disinfected, then cut and gently detached from underneath tissue. Biopsies were taken with biopsy punch and immediately placed into 10% neutral buffered formalin solution (Sigma). Surgical area was throughout disinfected with sterile gauze immersed into clorexyderm solution. Wound was sutured with suturing aids (Peters surgical) and 3M Vetbondt tissue adhesive (3M Animal Care Products). After surgery, mice were given carprofen enriched-diet for pain management (Clear H_2O) and housed in separate cages. Mice were daily checked to ensure health status until the wound was completely healed.

2.1.2.4 Necropsy

Mice were euthanized through cervical dislocation or CO_2 suffocation. Inferior vena cava was cut and mice perfused with PBS 1X from the left ventricle of the heart. Tracheal PBS 1X perfusion was performed to eliminate residual blood from the lungs. Samples were fixed in a 10% neutral buffered formalin solution (Sigma) over night for histological analysis or snap frozen in liquid nitrogen.

2.2 Histological and immunohistochemical analysis

2.2.1 Whole mount of mammary glands

Mammary glands were spread onto superfrost slides (Thermo Scientific), allowed to dry and placed for 1 hour in Carnoy's fixative (methanol:acetic acid, 3:1). Tissues were stained O/N with Carmine Alum stain (Bio Optica), then de-stained with acidic alcohol for the desired amount of time. Fat pad was cleared in xylenes and slides were mounted with DPX mountant (VWR).

2.2.2 Hematoxylin and Eosin staining (H&E)

Paraffin embedded samples were sectioned at 5 µm with RM 2135 microtome (Leica) and placed onto superfrost slides (Thermo Scientific). Hematoxylin QS (Vector) and Eosin 1% (Bio optic) staining was performed according to standard protocols. Slides were analyzed at LMD 7000 microscope (Leica).

For the quantification of lung metastasis, 5 µm sections were taken every 150 µm and stained with H&E.

2.2.3 Immunohistochemistry analysis

5 µm sections were de-paraffinizsed, re-hydrated and antigen retrieval was performed in 0,09% (v/v) unmasking solution (Vector) for 30 minutes in a steamer. Endogenous hydrogen peroxidase activity was inactivated with 10% H_2O_2 solution (Sigma). After, sections were blocked with blocking buffer (10% serum in PBS 1X), incubated with primary antibody and then, washed twice, 5 minutes each, with PBS 1X. Incubation for 30 minutes with biotinilated antibody followed (Vectastain® ABC kit, Vector Laboratories). After antibody washing, sections were incubated with HRP conjugated antibody and revealed with DAB Peroxidase Substrate kit (Vector). Slides were contra-stained with hematoxylin QS (Vector), de-hydrated and mounted with DPX mountant (VWR). Slides were analyzed at LMD 7000 microscope (Leica).

Primary antibodies: HA (Roche, 11867423001) 1:200 dilution, MYC (Cell Signaling, 56055) 1:1000.

2.2.4 Immunoflurescence of FFPE samples

5 µm sections were cut from FFPE (formalin fixed paraffin embedded) samples onto superfrost slides (Thermo Scientific). Slides were de-paraffinized washing them twice in xylenes (Sigma) for 15 minutes and re-hydrated in EtOH. Then, antigen retrieval was perfomed and slides were permeabilized in 0.5% Triton X-100 diluted in PBS 1X for 45 minutes. Slides were washed three times in PBS 1X, each time 5 minutes. Blocking with 5% goat serum (Jackson ImmunoResearch) in PBS 1X for 1 hour followed. Primary antibodies were incubated at room temperature for 1 hour and a half. Slides were washed three times for 5 minutes in PBS 1X, then incubated with AlexaFluor® conjugated secondary antibodies (Life Technologies; dilution 1:1000) directed against the host species of the primary antibody for 1 hour at room temperature in the dark. Slides were again washed in PBS 1X, then stained with DAPI (Life Technologies; concentration 1 µg/ml) and mounted with 20% (w/v) Mowiol mounting medium. Slides were analysed at LMD 7000 microscope (Leica).

Mowiol mounting medium was prepared by the Histology Facility as follows: 5 g of Mowiol were dissolved in 25 ml of mQ water at 55 °C, overnight with constant shaking. After, it was aliquoted and stored at +4 °C.

Primary antibodies: Citokeratin 8, Endo-A (DSHB, TROMA-I) 1:200 dilution, Keratin 14 (Covance, PRB-155P) 1:1000 dilution.

2.3 Biochemical procedures

2.3.1 RNA extraction and qPCR analysis

2.3.1.1 RNA extraction

Snap frozen tissue was homogenized in a mortor. Frozen powdered tissue was collected with 800 µl ml of ice cold TRIzol® reagent (Ambion) and put into a 1.5ml eppendorf tube. Samples were incubated at RT for 10 minutes. Next, 200 µl of chloroform was added. Samples were vigorously shaken for 15 seconds and incubated at RT for 3 minutes. Samples were centrifuged at 15000 rpm, 15 minutes at 4°C. Aqueous phase was separated in a new tube and processed for RNA precipitation and purification. 500 µl of isopropanol (Sigma) and 1 µl of linear poliacrylammide (Sigma) were added. Samples were incubated at RT for 10 minutes, then centrifuged at 12000 rpm, 10 minutes, 4°C. Supernatant was removed and the pellet washed with 1 ml of 75% EtOH. Next, samples were centrifuged at 7500 rpm, 5 minutes, 4°C. Supernatant was discarded and pellet allowed to dry at RT. RNA was resuspended in 30 µl of RNAse free water.

2.3.1.2 Genomic DNA digestion

Contaminations of DNA were eliminated using TURBO DNA-free™ kit (Ambion). TURBO™ DNAse (2 Units/µl) was inactivated and RNA was further purified using RNeasy Mini kit (Qiagen).

2.3.1.3 Determination of RNA yield

RNA concentration was measured using Qubit® RNA Assay Kit (Invitrogen, Q32855). 500 ng of RNA were loaded on a 1% agarose gel to determine RNA integrity.

2.3.1.4 cDNA synthesis

200 ng of purified RNA were used for cDNA synthesis. The reaction was carried out with Super Script® III RT kit (Invitrogen) or Quantitect Reverse Transcription Kit (Qiagen).

2.3.1.5 qPCR

Quantitative PCR was performed using SYBR Green qPCR mastermix (Applied Biosystems) according to manufacturer's instructions in Lightcycler® 480 System instrument (Roche). PCR reaction program was as follows:

See Table 2.3 for complete list of primers. The fold changes in gene expression were calculated using the $\Delta\Delta$Ct method.

Step	Temperature (°C)	Time (s)	Cycles
Denaturation	95	300	1X
Denaturation	95	10	45X
Annealing	60	15	
Elongation	72	10	
Denaturation	95	5	1X
Annealing	65	60	1X
Denaturation	95	N/A	Continuous
Cooling	40	10	1X

Table 2.2: Detailed qPCR program.

	FW	RW
Snail 1	TCCAAACCCACTCGGATGTGAAGA	TTGGTGCTTGTGGAGCAAGGACAT
Rat Her2	TGTACCTTGGGACCAGCTCT	GGAGCAGGGCCTGATGTGGGTT
HA-Mad2	TCCCAGAAAGCCATACAGGA	GTCCCGATTCTTCCCACTTT
L37	TCTGTGGCAAGACCAAGATG	GACAGCAGGGCTTCTACTGG
Actin B	GCTTCTTTGCAGCTCCTTCGT	ACCAGCCGCAGCGATATCG

Table 2.3: Forward and reverse qPCR primer sequences.

2.3.2 Sequencing

2.3.2.1 Sample preparation

50 ng of cDNA were used to amplify fragments of interest. Primers and relative annealing temperatures are indicated in Table 2.3.
PCR products were purified using QIAquick PCR purification kit (Qiagen). 5 µl of PCR product were used to synthetize forward and reverse strand for sequencing (Big dye terminator, Invitrogen). Products were precipitated at 13200 rpm, 20 minutes, RT in 64 µl EtOH 100% and 16 µl mQ water. The supernatant was removed and the pellet was washed with 400 µl of EtOH 70%. After discarding the supernatant, pellet was air dried, then resuspended in 10 µl of mQ water. 4 µl of DNA suspension was added to 16 µl of deionised formammide and given for sequence to the in-house facility.

	FW	RW	Amplification parameters
rtTA	TGATTAACAGCGCATTAGAG CTGC	AAAGTGGGTCCGCGTACAG	60°C, 40 cycles
Kras	GTAAGGCCTGCTGAAAATG	GTGAATATCTTCAAATGAT	57°C, 40 cycles
Hras	AGAATACAAGCTTGTGGTG GTGG	CCTGTACTGATGGATGTC CTCG	57°C, 40 cycles
Nras	GGTCTCCAACAGCTCAGGTT GAAG	GTACCTGTAGAGGTTAATATC TGCA	57°C, 40 cycles

Table 2.4: Forward and reverse primers for sequencing with relative PCR conditions.

2.3.2.2 Sequence analysis

Sequences were analysed with 4Peaks software. Mutations were validated only when present both in forward and reverse strand.

2.3.3 Comet assay

2.3.3.1 Slide preparation

Superfrost Plus slides (Thermoscientific) were dipped in 1% liquid agarose (Sigma). The excess of agarose was removed and slides were dried at 37°C.

2.3.3.2 Primary tumor cells preparation

In order to detect DNA damage at single cell level, primary tumor cells - prepared as described in 2.4.2 - were cultured in 6 well collagen-coated plates (BD Biosciences) for 24h in MEBM media (Lonza). Media was changed and, after 24h, cells were collected by trypsinization (0.25% trypsin, Gibco). Trypsinisation was inactivated using DMEM (Lonza) 25 mM Hepes (Gibco), 10% Tet Free FBS (Clontech), 1% Pen/Strep (Gibco). Cells were incubated in DNase I (5 mg/ml. Roche) for 5 minutes. After centrifugation at 1000 rpm, 5 minutes, RT cells were resuspended in 1 ml of PBS 1X supplemented with heparin (4 µg/ml, Sigma). Cells were centrifuged again at the same settings and the supernatant was removed. Pellet was resuspended in 140 µl of 1% liquid low melting point agarose. Two drops of equal volume were seeded onto previously agarose coated slides, then, quickly covered with 22x22 coverslips and incubated at 4°C for 5 minutes.

2.3.3.3 Lysis

1 ml of Triton X-100 was freshly added to 100 ml of lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10). Coverslips were removed from slides and placed in this solution on a horizontal staining jar for 1 hour at 4°C.

2.3.3.4 Alkaline treatment

Slides were incubated for 40 minutes in electrophoresis solution (0.3 M NaOH, 1 mM EDTA). Solution was poured into electrophoresis tanks at 4°C one hour before use. Slides were placed onto the platform tank, immersed in solution, forming complete rows. Any gap was filled with blank slides.

2.3.3.5 Electrophoresis and neutralization

Electrophoresis was done at 25V for 30 minutes. After, slides were washed 3 times for 5 minutes in neutralizing buffer (0.4 M Tris HCl, pH 7.5).

2.3.3.6 Staining and storage

Slides were stained with Hoechst 1 mg/ml and analysed at LMD 7000 microscope (Leica). Slides were stored at 4°C in a moist chamber.

2.3.3.7 Comets analysis

Comets were analyzed using the OpenComet software. Results were plotted as percentage of DNA contained in the comet tail.

2.4 Cell culture

2.4.1 Primary mammary epithelial cell culture

Mammary glands from 9 weeks old, doxycycline naïve transgenic mice were digested and cultured as in Jechlinger et al., 2009 and Lee et al., 2007.

2.4.2 Immunoflurescence of 3D cell cultures

3D gels were digested for 30 minutes at 37°C with 2.5 µl/ml Liberase^{TM} research grade (Roche) and 2.5 µl/ml Collagenase type 3 (Worthington), washed with PBS 1X, and fixed for 10 minutes in 4% parafolmaldehide. Washes for 15 minutes in IF buffer 1X followed for three times. Blocking in IF 1X buffer plus 10% goat serum (Jackson Immunoresearch) followed for 1 hour. Then, gels were further blocked in IF buffer 1X plus 10% goat and 10 µg/ml goat anti-mouse $F(ab)_2$ fragments (Jackson Immunoresearch) for 30 minutes. Primary antibodies were incubated O/N at 4°C. Primary antibodies were washed three times for 20 minutes in IF buffer 1X; Alexa conjugated secondary antibodies (Invitrogen, 1:1000 dilution) were incubated for 1 hour at room temperature. Three IF 1X washes followed, then gels were further washed with PBS 1X. DAPI (Roche) was used at 0.5 ng/ml in the last IF 1X wash. Gels were mounted with the anti-fade agent Vectashield (Vector laboratories).

1X IF buffer: 130 mM NaCl (Sigma), 7 mM Na_2HPO_4 (Sigma), 3.5 mM NaH_2PO_4 (Sigma), 7.7 mM NaN_3 (Merck), 0.1 % BSA (Sigma), 0.2 % Triton X-100 (Sigma), 0.05 % Tween (Sigma), pH 7.4.

Primary antibodies: Lamin B1 (Abcam, ab16048) 1:1000 dilution, E-cadherin (Invitrogen, 13-1900) 1:400 dilution, ZO-1 (Invitrogen, 61-7300) 1:500 dilution, HA (Covance, A594-101L) 1:2000 dilution.

2.4.3 *Ex vivo* breast primary tumor culture

2.4.3.1 Primary tumor digestion

Primary tumors were collected from euthanized animals. Tissue was mechanically dissociated, incubated for 3 minutes with Red Blood Cells Lysing Buffer Hybri-Max (Sigma) and centrifuged at 1000 rpm, 5 minutes, RT. Pellet was incubated in 5 ml DMEM media (Lonza) 25mM Hepes (Gibco), 1% Pen/Strep (Gibco), 2.5 µl/ml Liberase^{TM} research grade (Roche) and 2.5 µl/ml Collagenase type 3 (Worthington) for 1h. After PBS 1X wash, tumors were incubated in 0.25% trypsin (Invitrogen) for 30 minutes. Trypsinizsation was inactivated using DMEM (Lonza) 25 mM Hepes (Gibco), 10% Tet Free FBS (Clontech), 1% Pen/Strep (Gibco). Cells were incubated in DNase I (5 mg/ml, Roche) for 5 minutes. Dissociated cells were resuspended in serum-free MEBM media (Lonza) with supplements (Lonza, CC-3150), filtered with 70 µm and 40 µm cell strainers, then counted (LabTek II chamber, Thermo Scientific) in preparation for injection into cleared fat pad.

2.4.3.2 Primary tumor 3D culture

Dissociated primary tumor cells were cultured in 3D matrigel (Trevigen Inc.). 10,000 cells were seeded in 100 µl cell:matrigel droplets. Cells were fed with MEBM media (Lonza). Media was changed every 48h. Cells were monitored for growth every 2/3 days.

2.4.4 Metaphase spreads

2.4.4.1 Cell preparation

Primary tumor cells were cultured in 6 well collagen-coated plates (BD Biosciences) for 24h in MEBM media (Lonza). Aphidicolin (3 µg/µl Sigma) block for 8-10h followed. After washout, cells were incubated O/N with colcemid (0.015 µg/µl). The next day, colcemid was added up to 0.05 µg/µl and cells were incubated for 2h.

2.4.4.2 Chromosome spreads harvesting

After mitotic shake-off, media was collected into a 14 ml corning tube. Cells were washed with PBS 1X and removed from the plate with 0.25% trypsin (Invitrogen) treatment. Trypsinization was inactivated using DMEM (Lonza) 25mM Hepes (Gibco), 10% Tet Free FBS (Clontech), 1% Pen/Strep (Gibco) and cells were collected into the same tube. Cells were centrifuged at 1000 rpm, 5 minutes, RT. Supernatant was removed and cells were resuspended in 1.5 ml of supplemented MEBM media (Lonza). Hypotonic solution (75mM KCl) was added very slowly, drop by drop, along the wall of the tube up to 10 ml. Cells were incubated at 37°C for 20 minutes. After, 3-4 drops of fixative solution (methanol:acetic acid 3:1) were added and tubes gently inverted. Cells were centrifuged at 1000 rpm, 5 minutes, RT. Supernatant was removed, leaving 1 ml behind in which to resuspend the pellet by flicking the tube. Fixative solution was added up to 10 ml; meanwhile, the tube was flicked from time to time to throughout allow cell fixation. Cells were centrifuged 1000 rpm, 5 minutes, RT.
The last step was repeated 2-3 times for a total of 3-4 fixations. After this step, cells were kept on ice.

2.4.4.3 Chromosome spreads preparation

Superfrost slides (Thermo scientific) were immersed in fixative solution and stored at -20°C O/N. Paper tissues were wet with water and put onto a heating block set at 80°C to produce hot vapors. After fixation, cells were resuspended in 100-500 µl of fresh fixative solution by flicking/vortexing the tube. Cells were splashed onto superfrost slides on top of the heating block. Fixative was let partially evaporate, then, new fixative solution was added on the slide. After, slides were dried on the heating block for 5-10 minutes and O/N at RT.
Slides used for M-FISH analysis were stored at RT for 10 days maximum; afterwards, they were kept in boxes with hygroscopic salts at -20°C.

2.4.4.4 M-FISH analysis

M-FISH analysis was performed using the 24XCyte kit from Metasystem (Zeiss) according to manufacturer's instructions. This technique was performed at the Chromosome Dynamics Core, The Welcome Trust Center for Human Genetics, Oxford, UK.

2.5 Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined using GraphPad Prism® software. P-values < 0.05 were considered statistically significant.

Chapter 3

Results

3.1 Generation of CIN mouse models of breast cancer

CIN plays a pivotal role in the progression and recurrence of malignancies in patients. However, as mentioned in the introduction, no mouse model that would combine chromosome instability in oncogene-driven breast tumorigenesis has, so far, been generated. In fact, mouse models with activating oncogenes often result in karyotipically stable tumors, a situation that does not entirely recapitulate the human disease (Duijf et al., 2012).

We crossed tetracycline inducible Mad2 mice (Sotillo et al., 2007) with mice that express either Her2 (Moody et al., 2002) or MYC (D'Cruz et al., 2001) in a MMTV (Mouse Mammary Tumor Virus (Gunther et al., 2002)) background, generating the TetO-Her2/TetO-Mad2/MMTV-rtTA and TetO-MYC/TetO-Mad2/MMTV-rtTA models. Herein, as point of nomenclature, these mice will be referred to as TI-Her2/Mad2 and TI-MYC/Mad2 - where TI stands for tetracycline inducible - or triple transgenic mice. In the same way, mice bearing only one of the transgenes will be mentioned as TI-Her2, TI-MYC and TI-Mad2 or double transgenic.
In these models the expression of both transgenes can be regulated specifically in the mammary gland - under the control of the MMTV promoter - by administration or withdrawal of doxycycline. Doxycycline, bound to the rtTA transcription factor, allows transgene expression; contrariwise, absence of this compound prevents exogenous gene transcription, thereby shutting off the production of transgenic proteins (Figure 3.1). The latter is a key point in this study, in particular considering oncogenes as the driving force of tumor formation. In fact, targeting oncogene dependence is the rationale behind current targeted therapies and this system, in mice, offers the possibility of perfectly mimicking the actual therapeutical protocol administered to patients. In this respect, it is of worth pointing out that only drugs specific for the Her2 receptor have been developed, while c-MYC is still an "un-druggable" oncogene.

Therefore, the TI-Her2/Mad2 and TI-MYC/Mad2 models, in comparison to TI-Her2, TI-MYC and TI-Mad2, gives us the possibility to:

- Faithfully recapitulate human disease
- Study the process of primary tumor formation in the presence of doxycycline
- Mimic targeted therapy, upon doxycycline withdrawal
- Investigate the influence of Mad2 overexpression in the primary lesion and in the relapse process

Mouse line	Description	Reference
TetO-HA-Mad2	Mice bearing the Mad2 cDNA fused with an HA tag. TetO repeats are cloned upstream the transgene	(Sotillo et al., 2007)
TetO-Her2	Mouse line expressing an activated form of the Her2 oncogene under doxycycline control	(Moody et al., 2002)
TetO-MYC	Mouse line over-expressing exon 2 and 3 of c-MYC human oncogene	(D'Cruz et al., 2001)
MMTV-rtTA	Mouse line where the rtTA is under the control of the Mouse Mammary Tumor Virus promoter	(Gunther et al., 2002)

Table 3.1: Description of mouse lines. Each mouse line used in these experiments is briefly described and original papers cited as reference.

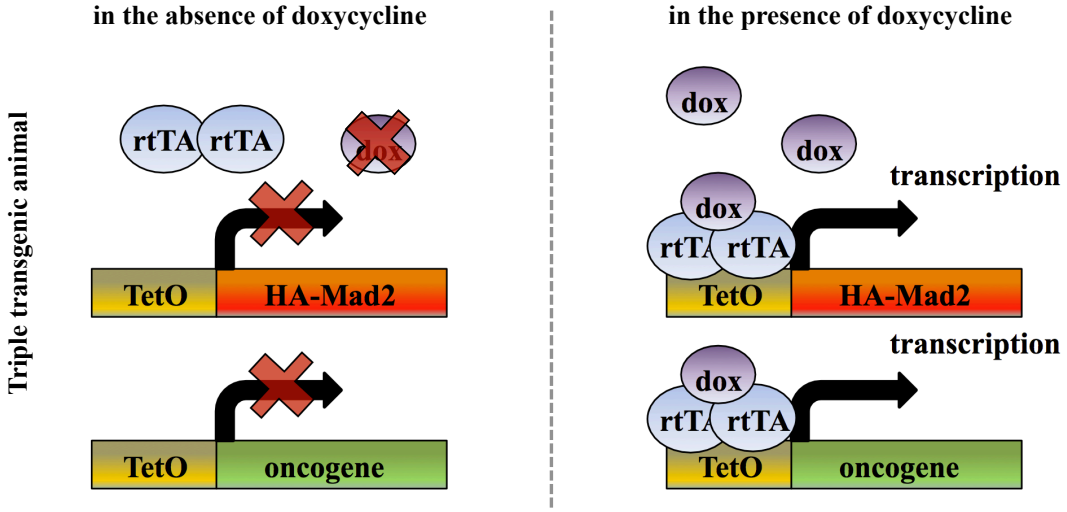

Figure 3.1: The Tet-On tetracycline inducible system. The tetracycline inducible system is made of two components: the rtTA transcription factor - under the control of the tissue specific MMTV promoter - and the TetO repeats cloned upstream the transgene of interest. Only in the presence of doxycycline (right panel) the rtTA dimer is able to bind to the TetO repeats and drive transgene expression. In our case, Her2 or c-MYC are the oncogenes used to drive tumor formation.

3.2 Mad2 over-expression is detrimental to primary tumor formation

3.2.1 Mad2 over-expression delays mammary gland tumorigenesis *in vivo*

In order to compare primary breast tumor latency, cohorts of experimental and control mice were raised. TI-Mad2, TI-Her2 and TI-Her2/Mad2, TI-MYC and TI-Mad2/MYC mice were generated and considered as experimental cohorts. Transgenic virgin females were switched to a doxycycline-enriched diet after complete development of the mammary gland (9 weeks of age), thus allowing transgene expression. Mice were weekly monitored to determine the appearance of malignant nodules.

Along with those mice, several control cohorts were raised. Transgenic mice bearing the same genotype as above mentioned animals, were fed doxycycline free food to exclude trans-

gene leakiness. To rule out any doxycycline effect and uncontrolled transgene activation by an endogenous mechanism, mice bearing only the rtTA module or the TetO repeats fused with a transgene were aged and fed with doxycycline impregnated food. None of these mice (n=51) developed mammary gland tumors in the timeframe considered for experimental purposes (500 days). Thus, this result confirms that the tetracycline inducible system used is these mice is tightly controlled and activated only in the presence of doxycycline.

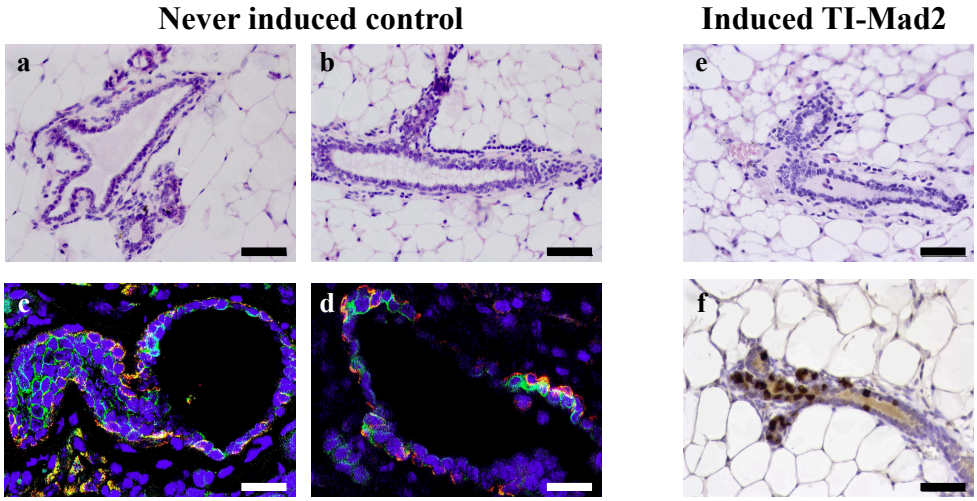

Figure 3.2: Mammary glands from control and induced TI-Mad2 mice do not develop primary tumors. a,b) Panels showing H&E staining of mammary glands from aged control mice. Normal mammary glands are characterized by the presence of ducts and terminal end buds, surrounded by adipose tissue. c,d) Immunofluorescence (IF) against K8 (green) and K14 (red) in normal mammary glands, showing distinct luminal and basal polarization and the characteristic organization of double layered epithelium. e) H&E staining of doxycycline TI-Mad2 animal. At aged-matched samples (500 days), TI-Mad2 mammary glands show the same structural organization than controls. f) HA IHC of TI-Mad2 induced mammary gland showing HA-Mad2 transgene expression. H&E and HA IHC scale bar 50 µm; IF scale bar 27.8 µm.

TI-Mad2 (n=20) mice do not develop mammary gland tumors upon doxycycline induction in the timeframe of analysis (500 days), suggesting that Mad2 over-expression by itself is not sufficient to drive breast cancer initiation, at least under the control of the MMTV promoter. In fact, it was surprising to observe that, Mad2 over-expression *in vivo* induces a delay in primary tumorigenesis, irrespective of the oncogenic background (Figure 3.3). TI-Her2/Mad2 and TI-MYC/Mad2 mice develop primary tumors with statistically significant longer latency compared to TI-Her2, TI-MYC animals. These results are in contrast with what has been previously published in a CIN model of lung tumorigenesis (Sotillo et al., 2010) highlighting that, in mammary epithelial cells, Mad2 over-expression is detrimental for tumor initiation.

3.2.2 Mad2 over-expression affects tumor multiplicity *in vivo* in Her2 driven breast tumorigenesis

Given the previous findings, we sought to understand if Mad2 over-expression, in combination with an oncogene, could not only, delay primary tumorigenesis but also affect tumor multiplicity *in vivo*. Female mice have ten mammary glands and, potentially, all of them could develop tumors with the same probability. However, this was rarely observed in our cohorts (only one case in a total number of 190 experimental mice), with the average number of tumors per animal being lower. Therefore, we counted the number of primary tumors per

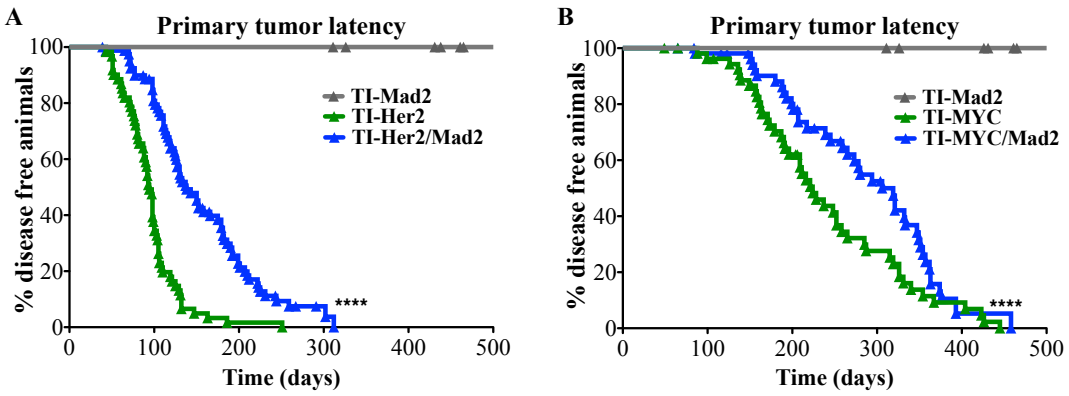

Figure 3.3: Mad2 over-expression delays breast cancer formation, irrespective of the oncogenic background. Kaplan Meyer plot of primary tumor latency in TI-Mad2 (grey), TI-Her2 or TI-MYC (green) and TI-Her2/Mad2 or TI-MYC/Mad2 (blue) lines. Panel A and B respectively. Timepoint 0 indicates when mice were set on a doxycycline diet (9 weeks of age). Each stair on the lines represents a positive event, that is, a mouse that developed a primary tumor, plotted against time (days). TI-Mad2 mice did not develop primary tumors in this timeframe. Mice over-expressing Mad2 in combination with an oncogene, have a delayed tumorigenesis compared to mice bearing the oncogene alone (TI-Her2 median latency 94 days, n=61 and TI-Her2/Mad2 median latency 138 days, n=68; TI-MYC 224 days, n=46 and TI-MYC/Mad2 321 days, n=36. Mantel-cox test, p-value < 0.001)

animal arising among double transgenic mice and compared it with the triple transgenic counterpart. Mad2 over-expression did affect tumor multiplicity when in combination with the Her2 oncogene. TI-Her2 mice developed an average of 5.2 tumors per mice, (n=53), while TI-Her2/Mad2 mice had, a significantly lower number of tumors (3.984) per animal (n=62; t-test, p-value=0.0022). In the case of MYC, mice develop in average 1.7 primary tumors (n=45) and in the combination of MYC with Mad2 the number is similar (1.5; n=30). The comparison between these two cohorts did not results in statistical significance (t-test, p-value>0.05).

All together, this result and the fact that Mad2 strongly delays breast cancer, highlights the tumor suppressive role of Mad2 in mammary gland tumorigenesis, even if evident only in the Her2 oncogene background. Why the same effect was not observed in case of MYC is not fully understood. However, one could hypothesize that in the case of MYC driven tumorigenesis, the average number of tumors per animal (1.7) is, *per se*, low. Thereof, even if Mad2 over-expression could have an effect in suppressing tumor multiplicity, this would be very difficult to detect *in vivo*. However, we could also hypothesize that Mad2 effect is dependent on the oncogenic background. Both Her2 and MYC are known to induce cell proliferation when over-expressed. The combination with Mad2 is predicted to over-activate the checkpoint and prolong mitosis, which potentially induces cell death (see 1.2.2 and 3.2.3). If this process is constantly ongoing because of sustained transgenes over-expression, the tissue must find a balance between cell death and regeneration. In the Her2 background, constant cell loss may gradually lead to an exhausted tissue and, as consequence, decreased tumor multiplicity. When MYC is deregulated, instead, cells are already sentisized to apoptosis (Hoffmann and Liebermann, 2008). Therefore, the combination with Mad2 may not result in an additive effect, but simply leave invariated the MYC induced outcome.

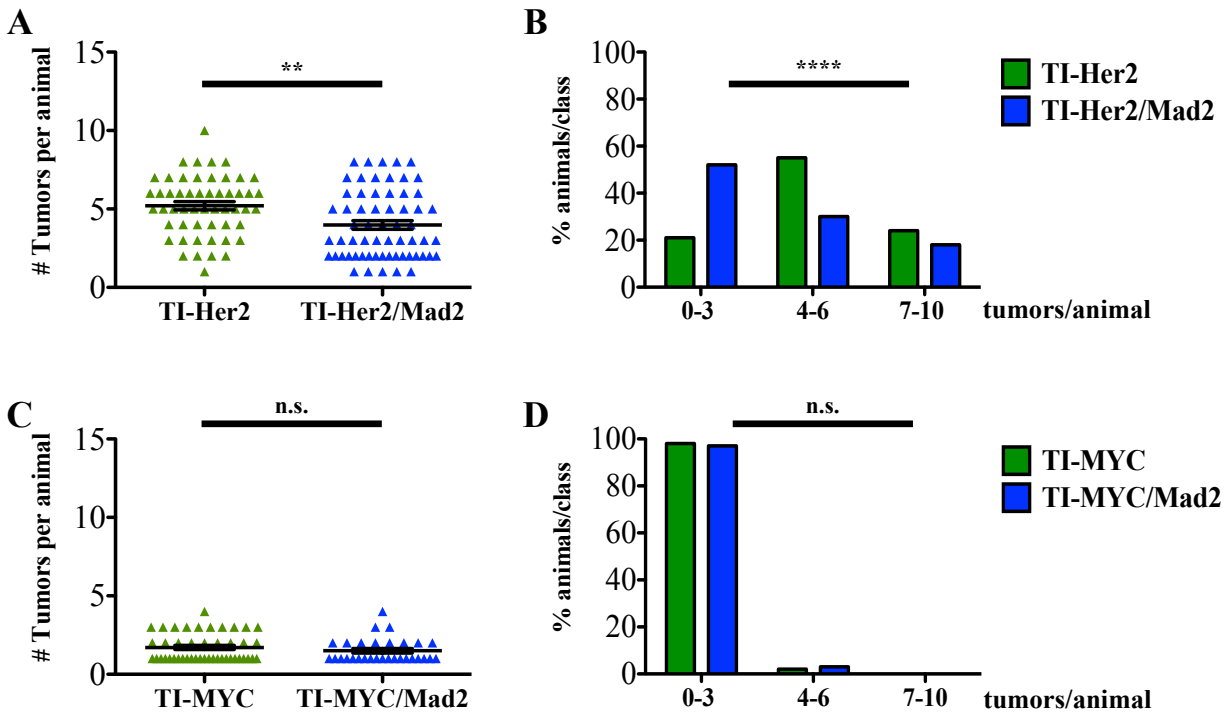

Figure 3.4: Tumor multiplicity is affected by Mad2 over-expression. A, C) Number of tumors arising in each experimental animal, TI-Her2 (n=53) and TI-MYC (n=45) (green), TI-Her2/Mad2 (n=62) and TI-MYC/Mad2 (n=30) (blue). Tumor multiplicity was affected only in the Her2 background. B,D) Distribution of mice in different classes depending on the number of palpable tumors at human endpoint stage. Difference in classes distribution was significant only in the Her2 background (Chi-square test, p-value < 0.0001).

3.2.3 Mad2 over-expression causes mitotic arrest *in vitro*

To better understand the delayed tumorigenesis induced by Mad2 over-expression we decided to investigate events happening at early time points of transgene induction. In fact, pre-tumorigenic stages are key determinants in further malignant progression and likely to help understanding the role of Mad2 in combination with a driving oncogene. To address this question, we resorted to a three dimensional cell culture system of primary mammary cells (Jechlinger et al., 2009). This system has been successfully used to study the effects of two potent oncogenes in culture, MYC and Kras, and it reproduces the observed phenotypes *in vivo*.

Primary mammary epithelial cells taken from virgin mice containing different transgene combination were grown in a matrigel based 3D culture system. Single cells develop organotypic acinar spheres within 6 to 8 days of culture and, after that, transgenes were induced by adding doxycycline to the culture media. 36h post induction, gels were fixed and stained with different antibodies. As controls, transgenic cells with identical genotypes were grown without the addiction of doxycycline.

Never induced control cells develop into a sphere-like hollowed structure, formed by a single layer of epithelial cells, characterized by a strict apical-basal polarization. At this time point, acini have reached a steady-state level where the optimal functional size is set and cells seldom divide to maintain it. In case cell division is happening, the polar spindle is parallel to the acinar lumen, allowing daughter cells to be integrated into the external rim layer, upon completion of mitosis (Jechlinger et al., 2009).

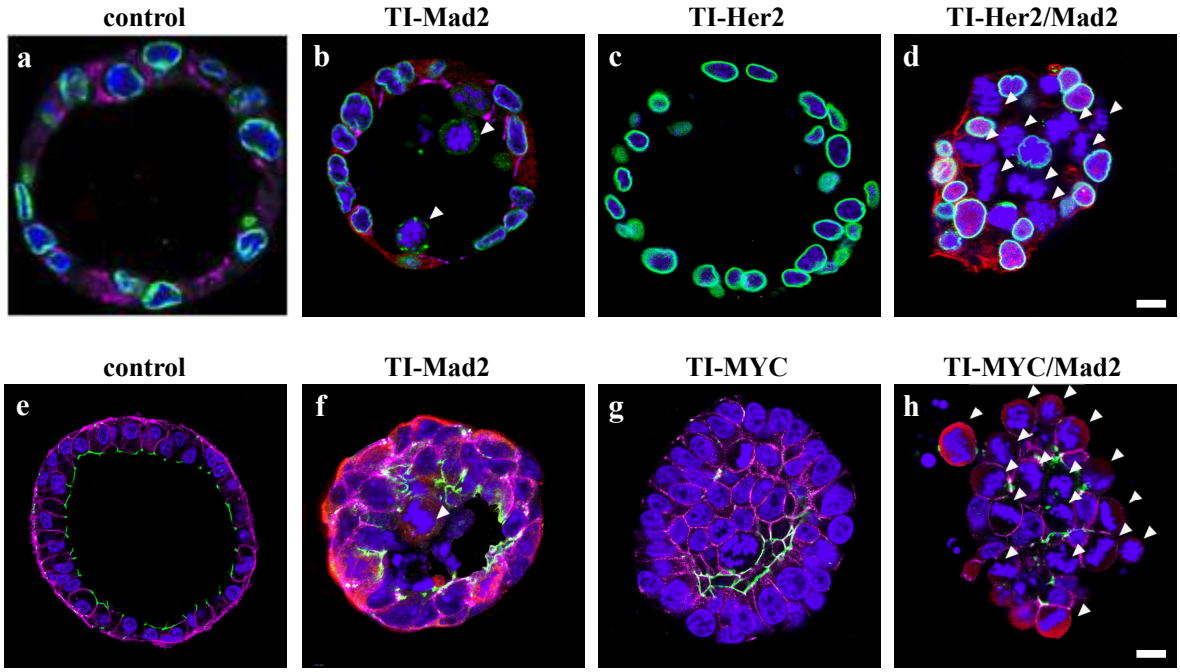

Figure 3.5: Mad2 over-expression causes mitotic arrest at early induction time point *ex vivo*. a) Mammary epithelial cells grown in 3D culture develop into polarized acinar spheres. b), f) Early 36h hours induction of Mad2 transgene induced a mild mitotic arrest without disrupting acinar organization (Panel b, K. Rowald). c) TI-Her2 induction did not show differences compared to controls; TI-MYC induced acini (g) developed into filled spheres. Combination of Mad2 with either oncogene (d, h) caused dramatic mitotic arrest 36 hours after adding doxycycline. Arrows indicate mitotic arrested cells. Immunoflurescence: DAPI (blue), red (HA-Mad2), Lamin B1 (green in a, b, c, d), ZO-1 (green in e, f, g, h), E-cadherin (purple). Scale bar 7 µm.

Over-expression of Mad2 alone does not appear to disrupt the acinar organization (Figure 3.5 b). When over-expressed, we observed cells arrested at the metaphase plate, suggesting that metaphase to anaphase transition was prolonged, consistent with Mad2 function in the SAC. Overexpression of Her2 for 36h did not show any difference when compared to uninduced cells (Figure 3.5 c). Acini were still organized and did not show an increase in the number of mitotic cells. When cells containing the MYC transgene were induced they started filling the acinar structure and gave rise to solid spheres. The different phenotypes observed between the two oncogenes, Her2 and MYC, in the 3D culture, is currently being investigated in the lab. It is possible that cells expressing Her2 need additional factors or stimuli from surrounding stroma cells that are lacking *in vitro*. Alternatively, oncogenic MYC could be responsible of a higher proliferative input *in vitro*, thus generating the difference with TI-Her2 cells.

TI-Her2/Mad2 induced acini showed a significant increase in the number of cells arrested in mitosis (Figure 3.5 d). This phenotype was further exacerbated when Mad2 was combined with MYC, where almost all the cells in every structure were mitotically arrested (Figure 3.5 h). A possible explanation is that the proliferation input given by the oncogenes pushes cells into division while at the same time, Mad2 over-expression blocks most of them at the mitotic stage. This is why we could observe this striking phenomenon only in the presence of Mad2. The stronger the oncogene influence, the more pronounced the phenotype was - so, in this respect, c-MYC exerts a more incisive influence at early time points.
In case of Mad2 overexpression alone or Mad2 in combination with an oncogene, mitotic

arrested cells appeared to fall inside the lumen of the acinar structure. We hypothesized that a prolonged mitosis could loosen cell-cell interactions, thereby promoting cell delamination (Dekanty and Milan, 2013) from the external rim. To further confirm these results and exclude the possibility of a culture artifact, data from our laboratory has shown that 4 day induction of Mad2 in the mammary gland also leads to mitotic arrest in vivo (Konstantina Rowald, unpublished data). Cell delamination and loss of epithelial contacts are known to be associated with anoikis (Taddei et al., 2012). In the mammary gland tissue, the ductal system may help the clearance of dead cells, and continuous cell elimination induced by Mad2 over-expression might generate a selection barrier a tumor should overcome before transformation occurs. In this context, Mad2 over-expression acts a tumor-suppressive gene and delays mammary gland tumorigenesis (K. Rowald, manuscript in preparation).

3.2.4 Mad2 over-expression promotes tumor heterogeneity

Since we observed differences in tumor latency of mice bearing the Mad2 transgene, we next wanted to examine if Mad2 could give rise to different tumor types. For this purpose, formaline fixed, paraffin-embedded tumors taken from mice at human endpoint stage, were analyzed for histopathological classification by a mouse histopathologist (collaboration with DMV Dott. Vittoria Castiglioni, Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria Facoltà di Medicina Veterinaria, Università degli Studi di Milano).
Primary tumors developed in TI-Her2 mice (n=18) are mostly solid multinodular carcinomas (60%), while the remaining ones are representative of microacinar (20%) and papillary (20%) subtypes.
TI-Her2/Mad2 malignancies (n=33) have a broader histopathological spectrum (Figure 3.6), with 38% being solid multinodular, 14% microacinar carcinomas, 41% tubulo papillary and 7% cribiform.

Multinodular	Cancer arising from clearly distinct nodules which together form the tumoral mass
Microacinar	Distinguished by the growth of small adjacent but distinct acinar structure
Papillary	Infiltrating breast cancer, characterized by well defined margins and small projections
Cribiform	Perforated growth pattern, microscopic architecture of epithelial cells growing back to back without intervening stroma

Table 3.2: Description of solid mammary tumor subtypes.

In case of MYC driven tumorigenesis, we could not appreciate any difference in the histopathological distribution of analyzed samples. 88% of TI-MYC primary tumors (n=16) show a papillary phenotype, with the remaining 12% belonging to the solid carcinoma type. When MYC is expressed in combination with Mad2, all the samples (n=24) fall uniformly into the papillary class (Figure 3.6 C).

3.2.4.1 Mad2 over-expression is detrimental to cellular fitness and selected against

Mad2 overexpression not only delays tumorigenesis but also significantly changes tumor morphology, suggesting a role of CIN in facilitating tumor heterogeneity. To better understand the patterns of Mad2 expression in the tumors, we took advantage of an HA tag in frame with the Mad2 transgene. This allows us to distinguish the exogenous Mad2 present in the

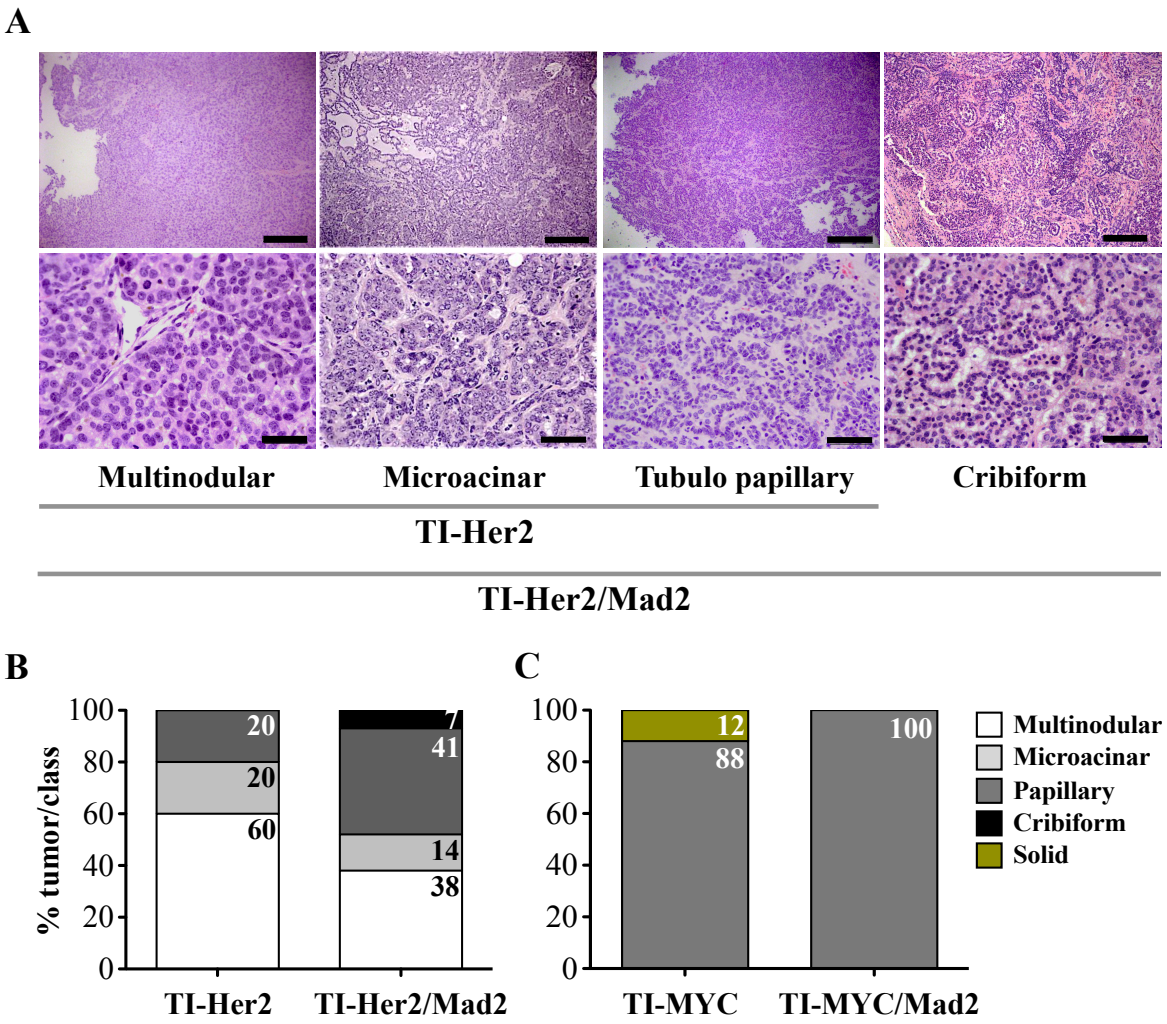

Figure 3.6: Histopathological classification in TI-Her2, TI-Her2/Mad2, TI-MYC and TI-MYC/Mad2 primary tumors. A) Heterogeneous histopathology (H&E staining) of TI-Her2 and TI-Her2/Mad2 primary tumors. B, C) Distribution of analyzed primary tumors per genotype according to the histopathological classification in the Her2 and MYC background. Upper panels scale bar 200 µm, lower panels 50 µm.

tumor.

Immunohistochemistry analysis of primary tumors at endpoint stage in the TI-Her2/Mad2 cohort revealed that 17.5% of the tumors do not show exogenous HA-Mad2 expression (n=10 out of 57 primary tumors analyzed). This result was further confirmed at the transcript level with RT-PCR in a smaller subset of samples. mRNA analysis and IHC of the same sample gave concordant results, suggesting that the Mad2 transgene was not expressed and the lack of protein was not due to post-transcriptional regulation of the mRNA (see figure 3.7).

Since Mad2 and Her2 transgenes are simultaneously regulated by the expression of rtTA and doxycycline, we next examined the expression of Her2 in those tumors that did not express Mad2. Interestingly, transgenic Her2 expression was induced and showed similar levels compared to tumors were Mad2 was also upregulated. (Figure 3.7). One could argue that levels of exogenous Mad2 induction may vary across mice. Therefore, the absence of HA-Mad2 could simply underline a very low, non-detectable, transgene expression. However, we could rule out this possibility comparing exogenous Mad2 expression between primary tumors and induced mammary glands from the same animal (see figure 3.8). As previously mentioned, tumor multiplicity in these mice is lower than the total number of mammary

glands. Consequently, within the same animal, mammary glands that developed a tumoral mass and mammary glands that - at the moment of euthanasia - had not developed a palpable nodule were compared. This analysis brought to our attention the fact HA-Mad2 negative primary tumors had matching samples showing, instead, both transgenes expression. This result further confirms the hypothesis that Mad2 over-expression is, indeed, tumor suppressive in the formation of primary breast cancer. Specifically in these cases - even if representing a minority of all analyzed samples - there is a strong selection against cells over-expressing Mad2, suggesting that, in a particular context, excessive CIN is not tolerated. Hence, in a specific tissue and environment, an optimum tolerated level of CIN should exist (Zasadil et al., 2013). Excessive CIN could compromise genome stability in a way that is incompatible with cell viability. These cells could be eliminated in an active process that is specifically selecting for those clones that have lower CIN levels. Milder CIN could promote the acquisition of genomic variability and, at the same time, preserving a certain degree of genome stability. This would allow the adaptation to changes in tumor environment without compromising cellular fitness.

Further corroborating this finding is the analysis of primary tumor latency sub-dividing the TI-Her2/Mad2 cohort into HA-Mad2 positive and negative tumors. HA-Mad2 negative primary tumors developed with a longer latency than the positive ones (209.5 days, n=10 and 133 days, n=47 respectively) and the difference between the two cohorts is statistically significant (Mantel cox test p value=0.0025).

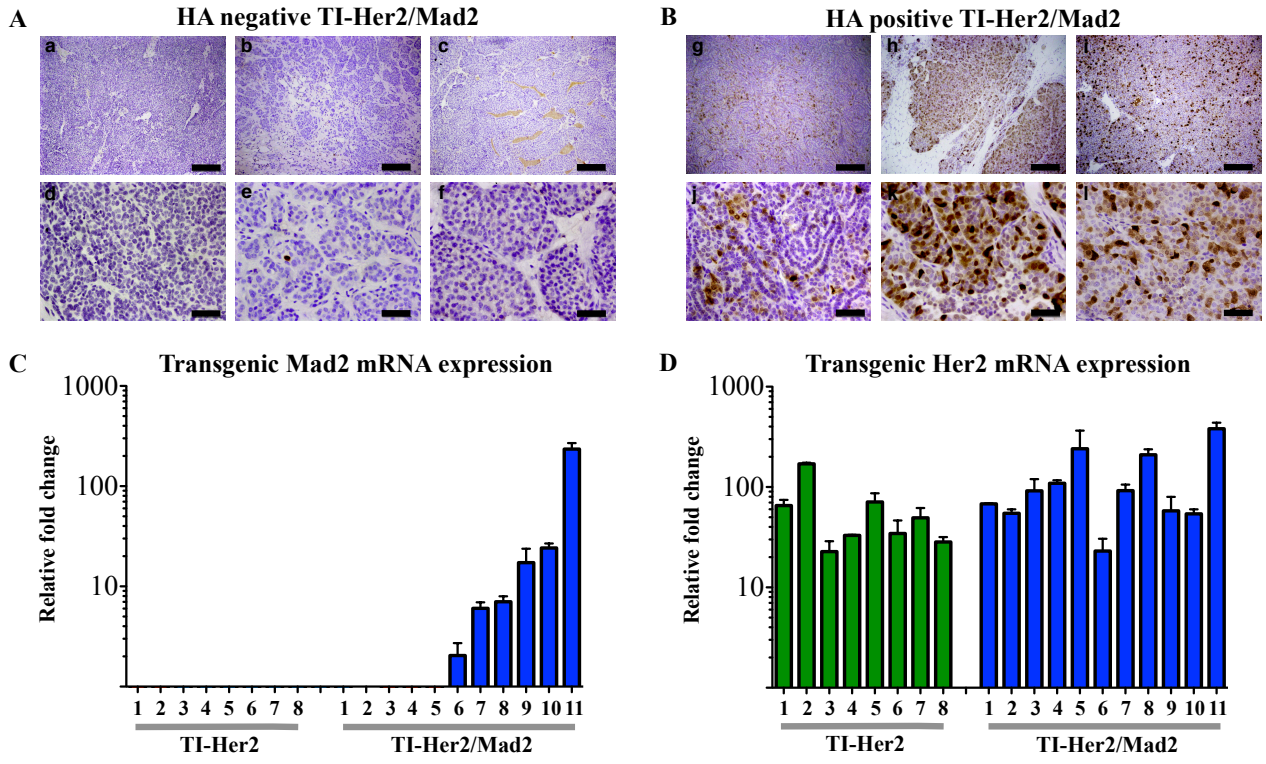

Figure 3.7: Mad2 transgene expression is downregulated in a subset of TI-Her2/Mad2 primary tumors. A) and B) Immunohistochemistry analysis performed on TI-Her2/Mad2 HA negative and positive primary tumors. Upper and lower panels show histological features of the same sample taken at different magnification. Upper panels scale bar 200 µm; lower panels scale bar 50 µm. C, D) qPCR analysis of Mad2 and Her2 transgenes expression in TI-Her2 (green) and TI-Her2/Mad2 (blue) in a subset primary tumors. The same samples were analysed for both transgenes expression (see corresponding numbers below both graphs).

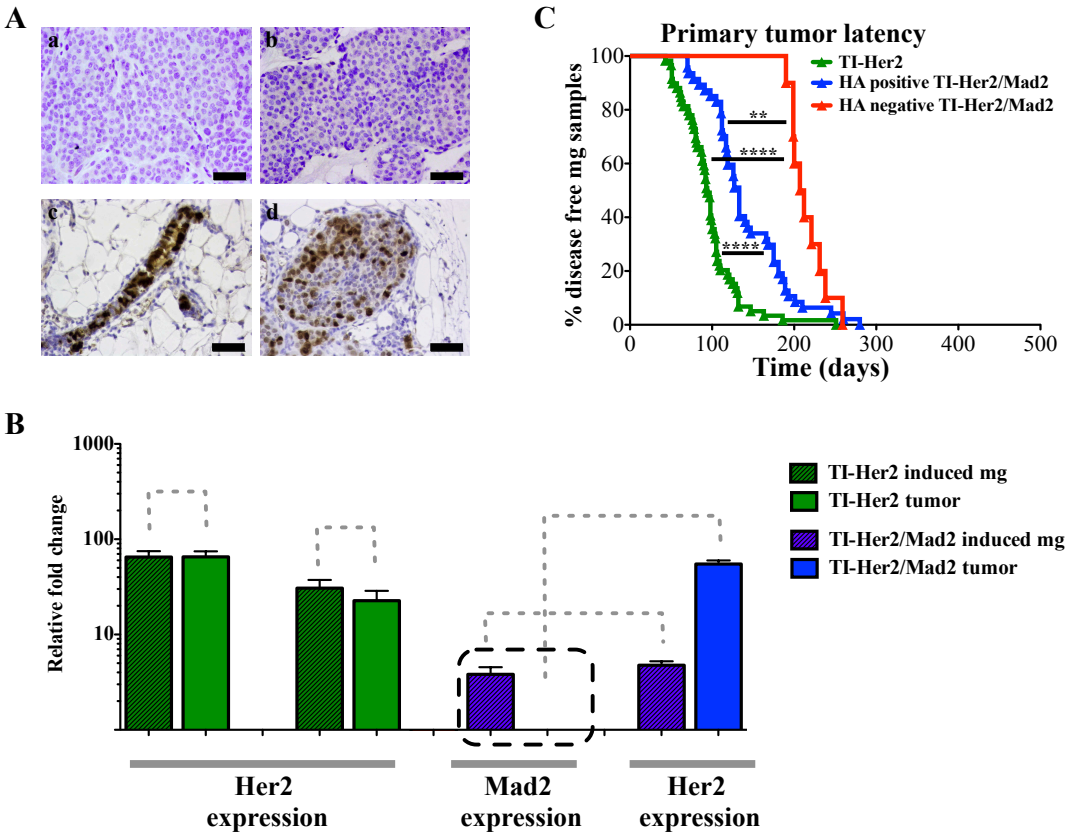

Figure 3.8: Exogenous HA-Mad2 expression is lost in a subset of TI-Her2/Mad2 primary tumors. A) HA IHC performed against two different primary malignancies (a,b) and induced mammary glands (c,d) with no palpable tumor from the same animal. Scale bar 50 µm. B) qPCR analysis for HA-Mad2 (blue) and Her2 (green) transgenes expression in matching induced mammary gland (striped pattern) and tumor samples (plain pattern). On the left Her2 trangene induction in TI-Her2 samples is shown. On the right part, HA-Mad2 and rat Her2 expression in induced mammary gland and primary tumor from the same animal are shown. TI-Her2/Mad2 primary tumor does not show exogenous Mad2 expression (dotted square). C) Kaplan Meyer plot of primary tumor latency in triple (HA positive TI-Her2/Mad2 blue, HA negative TI-Her2/Mad2 red) and double transgenic (TI-Her2 green) mammary gland (mg) tumor samples. In triple transgenic animals, the graph represents the latency of all primary tumor samples analyzed by HA IHC. In case of the TI-Her2 cohort, the latency of individual samples is plotted. The presence of a tumor was considered as an indirect proof of Her2 transgene expression.

In case of MYC driven tumorigenesis, IHC analysis of all TI-MYC/Mad2 primary tumor samples showed exogenous Mad2 expression (IHC analysis against the HA tag, data not shown). Therefore, it was not possible to carry out this type of analysis in this cohort.

3.2.4.2 Mad2 over-expression causes CIN and promotes higher missegregation rates

The previous finding prompted us to speculate that Mad2 over-expression may be the source of genetic variability that would, ultimately, be reflected in the tumor phenotype.
It has already been published that Mad2 over-expression *in vivo* leads to the formation of aneuploid tumors in mice (Sotillo et al., 2007, Sotillo et al., 2010). Moreover, human sample analysis correlated higher rates of CIN and poor patient outcome (Gutierrez and Schiff, 2011; Macrinici and Romond, 2010; Smid et al., 2010). In particular, most of these studies were carried out considering, as relevant subtype, Her2+ carcinomas. This is why we sought to compare the karyotype of TI-Her2/Mad2 versus TI-Her2 primary tumors.

M-FISH (T. Liehr, 2004) analysis was carried out to analyze the aneuploid status as well as chromosomal rearrangements. Six TI-Her2 and eight TI-Her/Mad2 primary tumors were subjected to M-FISH analysis by the Wellcome Trust Center for Human Genetics, Oxford (total number of painted metaphases was 189 and 172 respectively). A selection of representative M-FISH karyotypes in shown in figure 3.9.

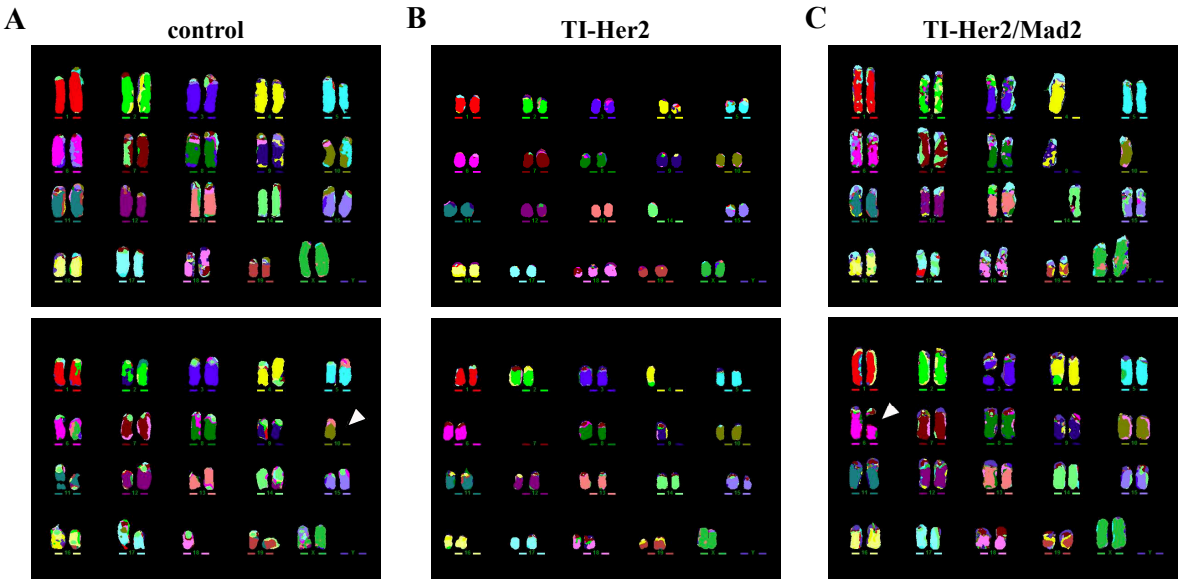

Figure 3.9: M-FISH analysis of control, TI-Her2 and TI-Her2/Mad2 primary tumors. A) Transgenic mammary epithelial cells dividing in the absence of doxycycline were used as controls to delineate possible technical bias. Karyotypes generally do not show any abnormalities, however occasionally chromosome losses can be appreciated (arrow). B) and C) M-FISH karyotypes of TI-Her2 and TI-Her2/Mad2 primary tumors. Both samples show chromosome gains and losses, as well as chromosome rearrangements. Only TI-Her2/Mad2 samples are characterized by DNA double strand breaks (arrow).

As it has already been mentioned, Mad2 is linked to aneuploidy both *in vitro* and *in vivo*. Therefore we first analyzed the karyotype from both genotypes (Figure 3.10 A). 69.66% of triple transgenic cells contain an abnormal chromosome number, while only 31.43% TI-Her2 ones deviate from the diploid status (Fisher's exact test, $p<0.0001$). However, karyotype analysis does not completely reflect the real "state" of aneuploidy within a tumor. In fact, a cell characterized by loss of one chromosome and gain of another one, would still figure out as being haploid. The number of chromosomes is still correct (in this case, 40) but this analysis would inevitably underestimate the real rate of chromosome missegregation. Therefore, we decided to specifically consider the fate of each chromosome in our samples to identify the likelyhood of mitotic errors in TI-Her and TI-Her2/Mad2 primary tumors.
Single chromosome missegregation analysis pointed out both inter and intra-tumor heterogeneity. Aneuploidy was not limited only to one specific chromosome, suggesting that Mad2 over-expression not only induces aneuploidy but whole CIN as well. It is of note to stress the notion that TI-Her2 samples are also characterized by whole CIN; however, this phenomenon can be observed at significantly higher rates in TI-Her2/Mad2 primary tumors.
As obvious causal factor, TI-Her2/Mad2 tumors tend to be more prone to missegregation errors. In cells with close-to-diploid chromosome number, 89% of TI-Her2/Mad2 cells contain, at least, one missegregation event and this is statistically significant if compared to double transgenic samples (79%; Figure 3.10 B). If we consider the number of missegregated chromosomes per metaphase, both genotypes may missegregate in a broad range going from

1 to 10 events in case of TI-Her2/Mad2 or even higher - up to 18, but only in sporadic cases - in TI-Her2 samples. Cells that contained 1 to 5 concomitant missegregated chromosomes were defined as bearing mild missegregation, while every cell having more than 5 was classifies as severe (Figure 3.10 C). One could hypothezise that Mad2 over-expression could promote higher mitotic defects, therefore skewing tumor cells towards a severe missegregation phenotype. However, this analysis did not point out any difference among TI-Her2 and TI-Her2/Mad2 primary tumors, suggesting that Mad2 over-expression is promoting higher missegregation rates but without promoting a severe phenotype. It might be that excessive missegregation events happening in the same cell are not compatible with cellular fitness or viability, and are, therefore, selected against in the tumorigenic process.
In fact, in both cases, there is a tendency towards the missegregation of two chromosomes per metaphase (see table 3.3), however, this is much more prominent in the presence of Mad2.

Number missegregated chromosomes/ Metaphase	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
TI-Her2	21	11	28	12	8	3	4	4	3	2	0	0	0.6	0	0	0	0.6	0.6	0.6
TI-Her2/Mad2	11	12	41	17	8	6	2	2	1	1	1	0	0	0	0	0	0	0	0
Control	80	10	5	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0

Table 3.3: Missegregation chromosome rates (%) in primary TI-Her2 and TI-Her2/Mad2 mammary gland tumors. Cells were divided into classes according to the number of missegregated chromosomes per metaphase. For each class, values in % are given across the different samples.

Recently, Benezra's group (Duijf et al., 2012) published a study of numerical chromosome aneuploiody performed on more than 43,000 human tumors. Data convincingly showed that tumors tend to loose, rather than gain, chromosomes. In particular, the loss is skewed towards small chromosomes but no inverse correlation was found for gains. One could think that acquisition of genetic material may lead to a phenomenon defined as proteotoxic stress, which impairs cellular growth in a way that is proportional to the amount of extra DNA (Torres et al., 2007). For this reason, gain would be a disfavored event compared to losses. However, preferentially gained chromosomes are not characterized by low coding gene content, hinting at the fact that, at least in established tumors, proteotoxic stress does not constitute a barrier for cancer survival and propagation.
On the other hand, chromosome loss has been shown to contribute to tumorigenesis via loss of tumor suppressor loci. Even though single copies might provide sufficient protein level (haplosufficiency), this event is of fundamental importance when one copy is already not functional. Therefore, loss of heterozigosity of tumor suppressor genes is an event likely to be fueled by chromosome loss.
In both TI-Her2 and TI-Her2/Mad2 samples, there was statistically significant difference comparing the average loss rate versus the average gain rate of samples from the same genotype (two samples test for equality proportions with continuity correction, p-value= 0.01286 and 0.00473 respectively). Considering the average of gain and loss per chromosome per genotype, chromosome 3 and 18 are gained at higher rates in TI-Her2 primary tumors (p-value=0.0292 and 0.0002 respectively). TI-Her2/Mad2 samples instead show a specific gain

of chromosome 6 (p-value=0.0001).
As far as losses are concerned, chromosomes 1, 2, 7, 8, 9, 11, 12, 15, 16 and 19 are lost specifically in TI-Her2 samples, while chromosome 4, 14, 18 are lost at higher rates in the TI-Her2/Mad2 genotype. It is worth to notice that chromosome 4 and 14 tend to be lost - rather than gained - also in TI-Her2 primary tumors, however, these events happen at statistically significant higher rates when in combination with Mad2 over-expression (Figure 3.10 E). Thus, it seems that events favoring Her2 driven tumorigenesis are exacerbated in triple transgenic mice. What is the advantage should depend, of course, on the genes encoded by these chromosomes.

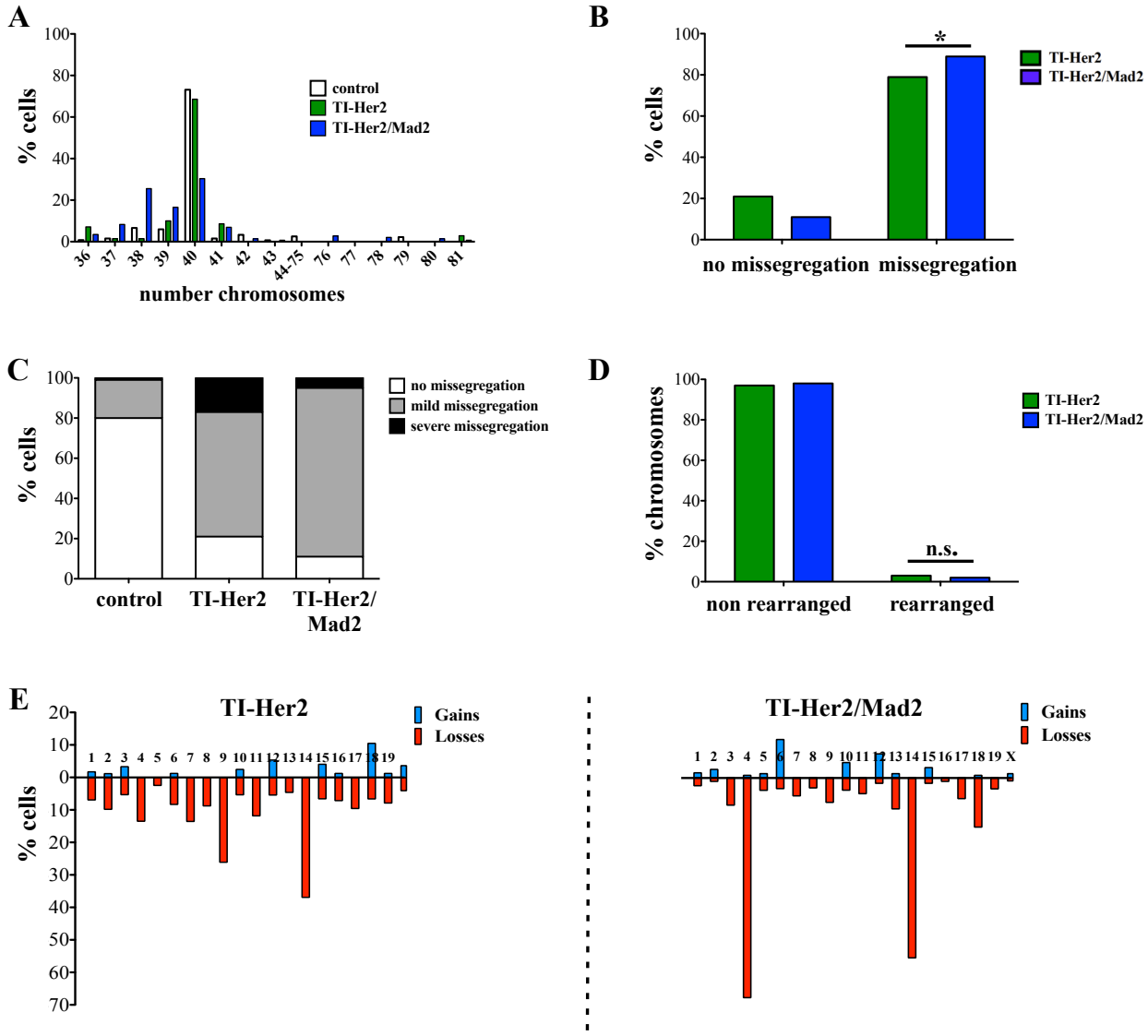

Figure 3.10: Mad2 over-expression causes higher rates of whole CIN. A) Distribution of cells (%) according to chromosome number content (control white, TI-Her2 green, TI-Her2/Mad2 blue). B) Percentage of cells with at least one missegregation event in TI-Her2 and TI-Her2/Mad2 primary tumors (Fisher's exact test, p-value=0.0246). C) Percentage of cells with no missegregation events (white), mild missegregation events (1 to 5, light grey) and severe missegregation events (more than five, black). The distribution is not different across TI-Her2 and TI-Her2/Mad2 primary tumors (Fisher's exact test, p-value> 0.05). D) Average percentage of rearranged chromosomes in TI-Her2 and TI-Her2/Mad2 is not statistically different across the two genotypes (Fisher's exact test p value>0.05). E) Average percentage of cells bearing gained/lost chromosomes in TI-Her2 and TI-Her2/Mad2 primary tumors.

Another key question is if Mad2 over-expression causes not only whole chromosome CIN but segmental CIN as well. For this reason, we analyzed the rates of translocated and derivative (that is a chromosome bearing rearrangements within itself) chromosomes. In this case, in order to correct for chromosomes gain/loss and 4n cells, rates were calculated with the actual number of chromosomes of analyzed samples (Figure 3.10 D). We did not find statistical differences between TI-Her2 and TI-Her2/Mad2 samples considering either derivative or translocation rates separately (Fisher's exact test). Total rearrangements rates did not give any statistical significance too (Fisher's exact test). In the same way, analysis of clonal events did not highlight any higher incidence of sporadic events in TI-Her2/Mad2 samples. This means that Mad2 over-expression in our model of breast tumorigenesis did not elicit higher segmental chromosome instability, which is already present in Her2 samples.

3.3 Mad2 over-expression accelerates tumor relapse

Tumor resistance is the major problem currently faced in the clinic. Patients are either inherently resistant to drugs or, in most of the cases, develop secondary resistance after treatment rendering unfruitful any already experienced therapy. Tumors will eventually resume growth in the same site where the primary tumor was found leading to relapse. Therefore, trying to understand molecular mechanisms underlying resistance is of utmost importance.
As previously mentioned, CIN is a common characteristic in tumor relapses and, when present in the primary tumor, it has been associated with shorter disease free survival, metastatic spread and therapy resistance (Smid et al., 2010). In this sense, unstable aneuploidy acts as a tumor-promoting event. Concurrently, it should also be remembered that high CIN rates might exert a detrimental effect on cellular fitness, up to be incompatible with cellular viability. Thereof, the outcome of different levels of aneuploidy will depend on a specific organism and cell type.
This is why targeting mitotic cell division has been used as therapeutical concept. The use of microtubule destabilizing agents (i.e. paclitaxel, vincristine, vinc alkaloids...), aimed at disturbing mitotic spindle formation and chromosome segregation, has been introduced and approved by the FDA since the last decade. Upon tubulin binding, microtubule-targeted polymerizing agents (MTPAs) perturb mitotic spindle stability, mitotic arrest in the cell cycle and G2/M transition (Bhalla, 2003). Several mechanisms of resistance have been discovered (Orr et al., 2003), underlying the fact that an optimal level of CIN should exist in order to promote tumor heterogeneity and drug resistant clone selection and avoid cell death. In breast cancer patients, MTPAs are administered as adjuvant chemotherapy treatment together with targeted therapy, when available (Garrison et al., 2007; Liberato et al., 2007). Of note, Her2+ breast cancers, whose progression is correlated with CIN (Smid et al., 2011), seem to be intrinsically resistant to this type of drugs (Tan et al., 2002); moreover, MTPAs are ineffective in genomic unstable relapses.

As piece of evidence from mouse models, Mad2 over-expression in combination with $Kras^{G12D}$ in the lung favored tumor relapse after oncogene withdrawal (Sotillo et al., 2010). Primary tumors and, even more, relapses were shown to be highly aneuploid. Therefore, CIN in primary lesions was the trigger promoting higher rates of recurrences and escape from oncogene addiction. MRI monitoring confirmed complete regression of primary tumors and recurrence in the same anatomical position.

In case of breast cancer mouse models, tumor size can be simply monitored by palpation. As explained in Materials and Methods, when primary tumors reached a size defined as human

endpoint, transgenic mice were set back to a normal food diet, thus switching off transgene expression. We first wanted to address if doxycyline withdrawal will allow complete primary tumor regression. This could have two different outcomes: on one hand breast tumors could regress completely and further mutations/events required for tumor relapse could happen during the regressed state. On the other hand, primary tumors may not completely regress after oncogenic shock, underlying the existence of therapy resistant clones already in the primary tumor.

We monitored oncogenic shock consequence in both c-MYC and Her2 mouse cohorts raised with or without Mad2 combination. In both cases, Mad2 over-expression in the primary tumor promoted tumor relapse with decreased latency and increased incidence. Since we found striking differences between the two oncogenic backgrounds, the results will be presented separately. The reason for this is the different outcome upon doxycycline withdrawal and preferred relapses mechanisms adopted to overcome transgenic protein expression.

3.4 The TI-MYC and TI-MYC/Mad2 mouse lines

3.4.1 Doxycycline withdrawal did not lead to complete primary tumor regression in the MYC background

It has already been shown that only 40% of c-MYC induced mammary tumors fully regresses after oncogene de-induction, while the remaining 60% does not regress completely (D'Cruz et al., 2001, Leung et al., 2011). Of these, 37% partially regressed and resumed growth within 1-2 months, while 23% showed no regression and remained dormant or continued to grow even upon doxycycline de-induction.
Since a different genetic background could affect these results, we followed a cohort of MYC induced animals to allow a comparison with mice also expressing Mad2. In our hands (n=28), 30% of mice that developed a primary mammary tumor, after oncogene withdrawal regressed to a non-palpable state then eventually relapsed after a median latency period of 75.3 days. 16% of the mice almost completely regressed (tumor diameter $<$ 5 mm) and then resumed growth (AR+G) after a median latency of 67.6 days. The largest group, representing 54% of the mice, showed incomplete regression (IR+G) and resumed growth after 50.4 days. This is in line with previously published data, highliting the fact that, after oncogene withdrawal, the majority of MYC-driven breast carcinomas do not regress to a non-palpable state suggesting that they are already independent of the driving oncogene.

In contrast, tumors expressing a combination of MYC and Mad2, (n=18) never completely regressed after deinduction of these transgenes. In this model, 25% had a considerable reduction in tumor size before starting to grow again (AR+G), while the remaining 75% showed only a minor reduction (IR+G) (median re-growth latency 47.8 days and 31.5 days respectively).
CIN, in the MYC background, is preventing primary tumors from complete regression in 100% of the cases. This underlies the existence of oncogene-independent clones already in the primary tumor, an event that is even more prominent in TI-MYC/Mad2 mice. Hence, Mad2 over-expression favored this process and rendered unfruitful any response to mimicked targeted therapy.
There was no statistical significance in relapse latency between the two cohorts (see figure 3.11 B). This was to be expected, since in both cohorts, no complete primary tumor regression occurs. However, time required for TI-MYC/Mad2 AR or IR (47.8 days and 31.5 days respectively) tumors to resume growth was significantly shorter than in the TI-MYC counterpart (AR TI-MYC 67.6 days, IR TI-MYC 50.4 days. Two way anova).

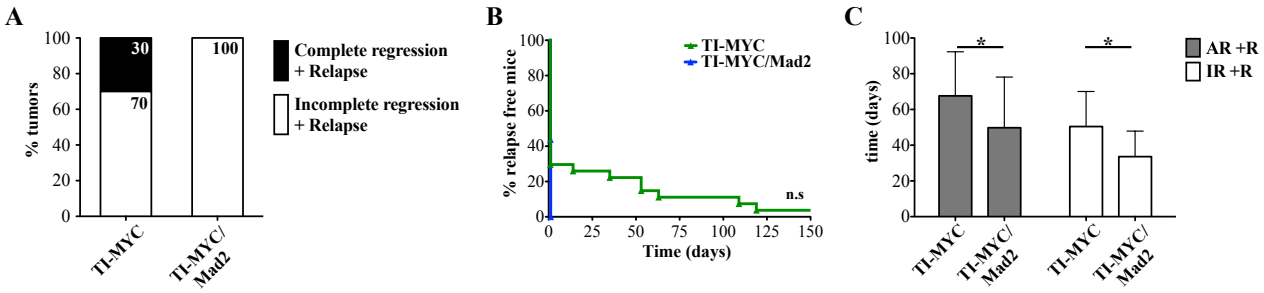

Figure 3.11: Doxycycline withdrawal in MYC-driven tumorigenesis lead to incomplete tumor regression. A) After doxycycline withdrawal, 30% of TI-MYC primary tumors (n=28) regressed to a non palpable state while the remaining 70% never completely regressed. None of TI-MYC/Mad2 mice completely remitted primary malignancies (n=18). B) Kaplan Meyer plot of relapse latency in TI-MYC (green) and TI-MYC/Mad2 mice (blue; Mantel-Cox test, p-value>0.05). The blue line close to the y axis represents 100% of the events for the TI-MYC/Mad2 cohort, which has no relapse latency since primary tumors never reached complete regression. In case of TI-MYC malignancies, 70% of the events is represented by the green line close to the y axis (AR and IR tumors). Relapse latency (days) for TI-MYC samples that completely regressed is afterwards plotted. C) Time (days) required for almost completely (AR, grey) or incompletely regressed (IR, white) tumors to resume growth (R) after doxycycline withdrawal.

3.4.2 Mechanism of relapse in MYC driven breast tumorigenesis

Previous studies in TI-MYC breast carcinogenesis (D'Cruz et al., 2001, Leung et al., 2011) have highlighted that primary tumors acquire independence from the driving oncogene by preferential mutations in the Kras2 endogenous locus. 80% of tumors that did not show sign of regression after removal of c-MYC carried a mutation in Kras2 (Leung et al., 2011). Alternatively, other members of the Ras family, Hras or Nras were affected whenever mutations in Kras2 were not detected (D'Cruz et al., 2001).

Therefore, we sequenced the Kras endogenous locus in the TI-MYC/Mad2 non regressing primary tumors, and observed that it was mutated in 37.5% (n=6) of the cases. Mutational analysis in both Hras and Nras endogenous loci was carried out in all the samples, and never these genes were found to bear spot mutations, unlike published data in TI-MYC model.
In 12.5% of the cases mutations occured in the rtTA region, allowing transgenes re-expression. In fact, in tetracycline inducible mice, a published mechanism of re-addiction to the initial driving oncogene occurs via point mutations of the rtTA element, converting the system from Tet-ON to Tet-OFF (Urlinger et al., 2000, Manfred Gossen, 1995, Winfried Hinrichs, 1994, Podsypanina et al., 2008).
The remaining samples (n=8), did not show any of these mutations. However, 37,5% of TI-MYC/Mad2 recurrences showed transgenes re-expression (confirmed by IHC analysis), despite not being associated with a mutation in the rtTA region. This highlighted the dependency of primary tumors to the initiating lesion and the possibility of finding different mechanisms to make this happen.

Histopathological analysis of TI-MYC (n=24) and TI-MYC/Mad2 (n=19) recurrences did not show striking difference in classification. In fact, in both genotypes, the two main types of tumor phenotype observed were papillary and EMT (TI-MYC: 62.5% and 37.5% respectively; TI-MYC/Mad2: 36.8% for both types). Only in case of TI-MYC/Mad2 non regressing tumors, some of them (26.4%) were classified solely as solid carcinomas. Nevertheless, in TI-MYC/Mad2 samples we could observe a correlative trend in tumor histopathology and the type of mutation occurring in recurrent tumors. In fact, EMT or spindled carcinoma samples were characterized only by mutations in the endogenous Kras gene or did not show

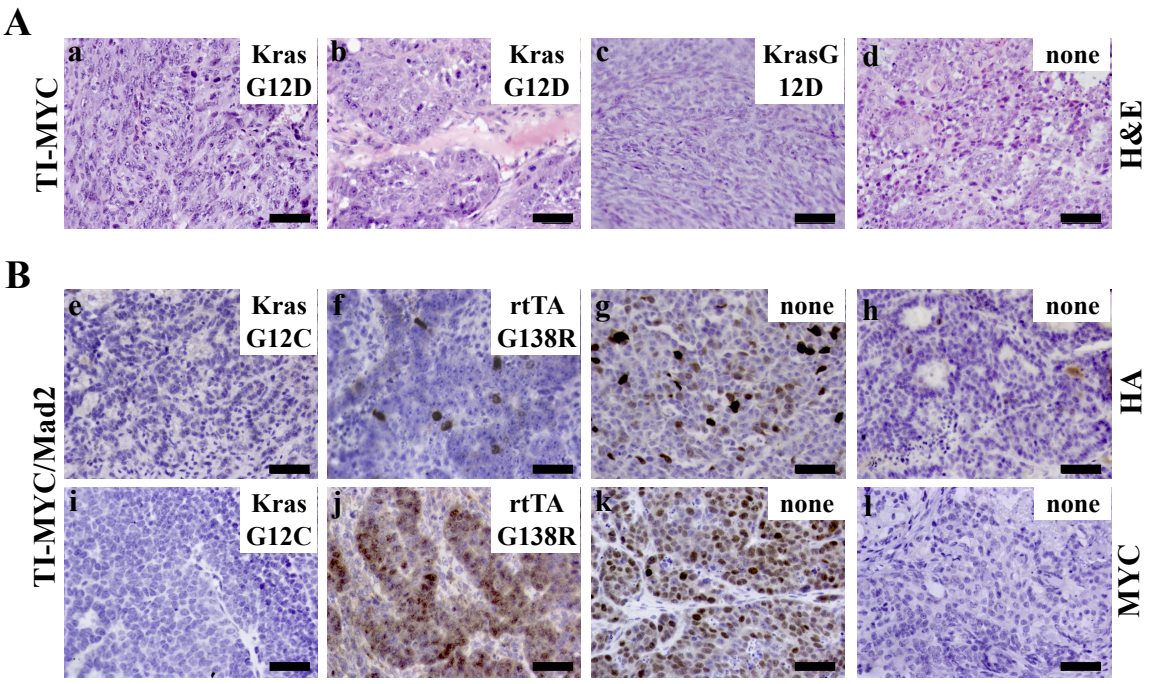

Figure 3.12: Mechanism of relapse and histopathology of TI-MYC and TI-MYC/Mad2 mouse lines. Small insets describe mutations found in the sample; if not present, no mutations were discovered in any of the following genes: Kras, Nras, Hras and rtTA (inset none). A) TI-MYC recurrences preferentially mutate the endogenous Kras oncogene (panel a,b,c). B) HA (panels e, f, g, h) and MYC (panels i, j, k ,l) IHC of TI-MYC/Mad2 non regressing tumors. Mutations can be found either in Kras or rtTA (e, f). 50% of TI-MYC/Mad2 samples did not bear any of these two mutations (g, h). Transgenes re-activation was observed also in absence of rtTA mutation (g). Scale bar: 50 µm.

any mutation in the analyzed loci. The papillary type was the most heterogeneous and could bear mutations either in the Kras or rtTA region or none of them. In none of these samples we could observe transgene re-expression in the absence of rtTA mutation (IHC performed against the HA tag and MYC exogenous protein). Contrariwise, we could not find any mutation in almost all solid carcinomas, the only exception being one example in the Kras gene. Only in this subclass transgene re-expression could occur even if no mutations were found in the rtTA region. The molecular mechanism leading to transgene re-expression in this case is still unknown to us.
The CIN status of these samples was not analysed, however we could hypothesize that Mad2 induced CIN conferred an advantage in relapse in different oncogenic backgrounds. A wider heterogeneity could give primary tumors the luxury of choosing alternative ways to achieve resistance to targeted therapy.

3.5 The TI-Her2 and TI-Her2/Mad2 mouse lines

3.5.1 Doxycycline withdrawal leads to complete primary tumor regression in Her2 driven tumorigenesis

We have previously described that Mad2 overexpression in the primary tumor in combination with oncogenic MYC leads to incomplete tumor regression after oncogene withdrawal. These results prompted us to analyze if this is a general phenomenon induced by Mad2. We took advantage of TI-Her2 and TI-Her2/Mad2 mice that developed primary tumors and

removed doxycycline from their diet when tumors reached a size of 20 mm in diameter. In this case, all tumors completely regressed to a non-palpable state, regardless of the genotype. However, this could mean not only that mammary glands effectively completely regress, but also that small cell populations could survive as non-palpable nodules. In fact, nodules become barely palpable through the mouse skin when they reach at least the size of 1 mm in diameter but cannot be detected with this method if smaller. To verify which hypothesis could be compatible with our mice, we analyzed freshly regressed mammary glands four weeks after no residual primary malignancy could be palpated any more. In this timeframe, it is likely to think that any information from the tissue will be a consequence of the primary tumor and the regression process, while events leading to relapse should occur later.

Both TI-Her2 and TI-Her2/Mad2 mammary glands looked completely regressed at this stage (Figure 3.13). Albeit the presence of scar tissue, in most of the analyzed tissues we could observed mammary ducts formed by an organized layer of cells immersed in fat.
IHC analysis of the HA tag in TI-Her2/Mad2 samples, did not highlight the presence of Mad2 expression, confirming that doxycycline withdrawal is effective and that there are no resistant clones arising in the primary tumor that re-express Mad2.

3.5.2 Mad2 induced CIN boosts relapse frequency and decreases relapse latency

After doxycycline withdrawal and complete primary tumor regression, mice were weekly monitored to determine the latency of tumor recurrence. Also in this case, primary tumors that over-expressed Mad2 are more prone to relapse (Figure 3.14). TI-Her2/Mad2 developed tumor recurrence with a significant shorter latency (109 days, n=18) than TI-Her2 mice (155 days, n=13).

Mad2 over-expression not only accelerates tumor relapse, but increases the frequency at which relapse event are observed *in vivo*. In our cohort, 28% of TI-Her2 mice are still tumor free in the timeframe of analysis (500 days) while in TI-Her2/Mad2 mice, the incidence of tumor relapse is 94.7%. Hence, in our mouse models, Mad2 over-expression in breast primary tumors helped the selection of drug resistant clones and correlated with worse prognosis, as it has been shown in human patients.

3.5.3 Relapse histopathology and mechanism of relapse in the Her2 oncogene background

It has already been described that TI-Her2 relapses are histologically characterized by an EMT (epithelial mesenchymal transition) phenotype (Moody et al., 2005). Cells have a well distinguished elongated shape, underlying loss of cell polarity, cell-to-cell adhesion and gain of migratory and invasive properties. We were able to reproduce the above mentioned data in our cohort of TI-Her2 mice. All the analyzed relapse samples have the characteristic EMT phenotype (Figure 3.16 A).
TI-Her2/Mad2 relapses (n=17), instead, can be divided, according to histological analysis, into two classes: 41.17% have an EMT phenotype, while 58.83% are characterized by a solid carcinoma classification (Figure 3.16). The latter is the only one found in human patients. Therefore, at least in about half of the cases, the combination of Mad2 and Her2 over-expression in the primary tumor lead to a relapse phenotype that more closely resembles the human situation.
It is known that Her2 tumors relapse via an up-regulation of the Snail1 transcription factor, a master EMT regulator (Moody et al., 2005). As in the TI-Her2 case, EMT-like relapses

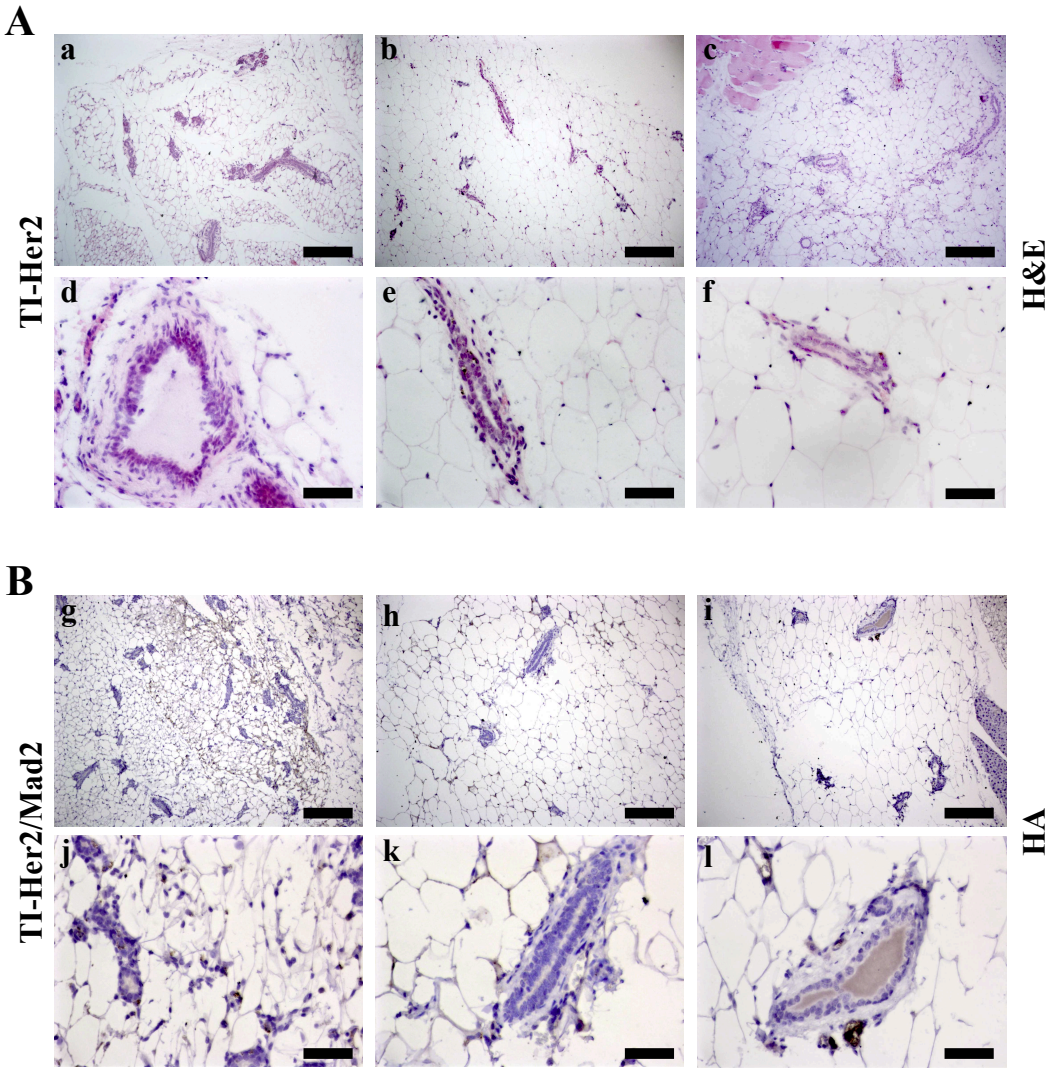

Figure 3.13: TI-Her2 and TI-Her2/Mad2 primary tumors regress completely to a non palpable state upon doxycycline withdrawal. A) H&E staining of 4 weeks TI-Her2 de-induced mammary glands. B) HA IHC against the exogenous Mad2 protein in TI-Her2/Mad2 regressed mammary glands (4 weeks de-induction). Scale bar: upper panels 200 µm and lower panels 50 µm.

from the TI-Her2/Mad2 cohort are characterized by up-regulation of the Snail1 trascription factor (Figure 3.15 A). Since EMT relapses have the same characteristics in both genotypes, we wondered what would be the peculiarity within the remaining half of TI-Her2/Mad2 relapses.

First, we decided to verify whether these samples showed re-addiction to the oncogenic pathway that fuelled primary tumor formation in first place. As explained in the introduction, patients become resistant to targeted therapy. However it has been demonstrated that tumor cells preferentially acquire mutations that bypass drugs mechanism of action, via a molecular mechanism that alternatively keeps active the signaling from the primary oncogenic lesion.

To test this, we first performed HA IHC against the exogenous Mad2 protein. 75% of solid relapses showed expression of this transgene and this result was further confirmed by qPCR analysis of both HA-Mad2 and Her2 transgenes (Figure 3.15). EMT-like relapses never had transgene re-expression in either genotype.

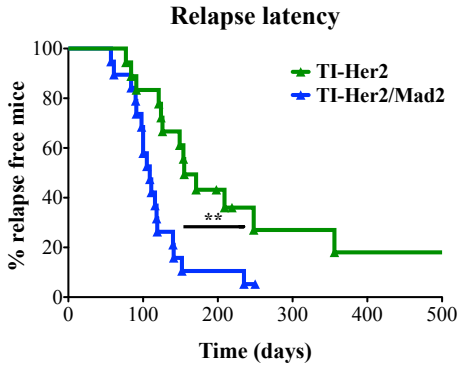

Figure 3.14: Relapse latency in TI-Her2 and TI-Her2/Mad2 cohorts. Time point 0 on the x axis indicates the moment when primary tumors reached humane endpoint size and mice were weaned from doxycycline. Each stair on the blue (TI-Her2/Mad2) or green line (TI-Her2) represents a mouse that developed a relapse. TI-Her2/Mad2 had a significantly shorter relapse latency compared to TI-Her2 mice (Mantel-Cox test, p-value= 0.0034).

Since the easiest explanation for transgene re-expression in the Tet system (see 3.4.2) is the mutation of the rtTA element, we performed mutational analysis in recurrent tumors. This revealed the presence of point mutations in the rtTA in all - but one - solid Mad2/Her2 relapses with transgene expression, while EMT looking ones did not (Figure 3.16).
Only one solid TI-Her2/Mad2 relapse was not affected by the rtTA mutation and, consequently, did not show any transgene re-expression. This is, indeed, the only example of this kind of relapse in our cohorts.
As conclusion, the combinatorial expression of Her2 and Mad2 in primary tumors promoted relapses that, in roughly half of the cases, mimic patient's situation in the clinic. Thus, due to the tetracycline system we are using, mutation of the rtTA module causes re-addiction to the oncogenic pathway causative of the primary tumor. This is often a reason why targeted therapy is ineffective in human patients.

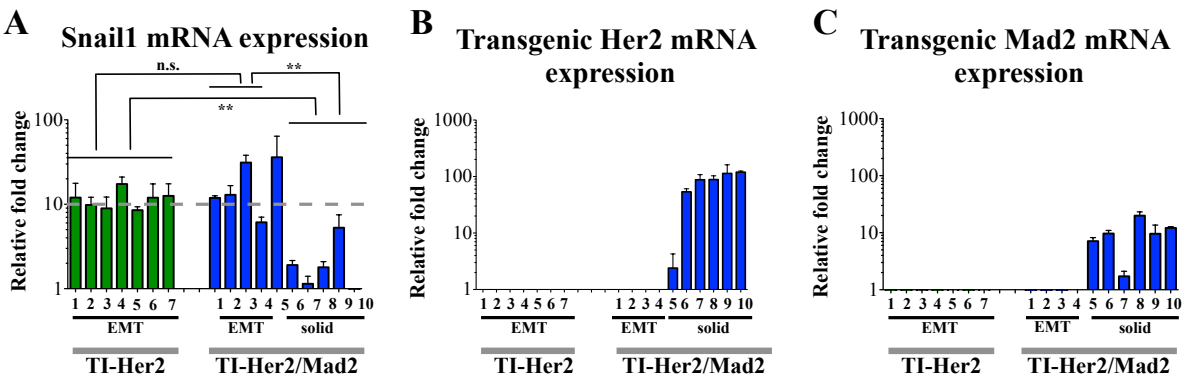

Figure 3.15: qPCR analysis of Snail1 and transgene expression in TI-Her2 and TI-Her2/Mad2 relapses. Analysis of a subset of relapses from TI-Her2 (green) and TI-Her2/Mad2 (blue) cohort; the same sample was analysed for all the above mentioned genes. A) qPCR Snail1 expression in TI-Her2 and solid or EMT-like TI-Her2/Mad2 relapses. EMT samples have significant higher expression (t-test). B and C) Fold change values of Her2 and HA-Mad2 transgenes.

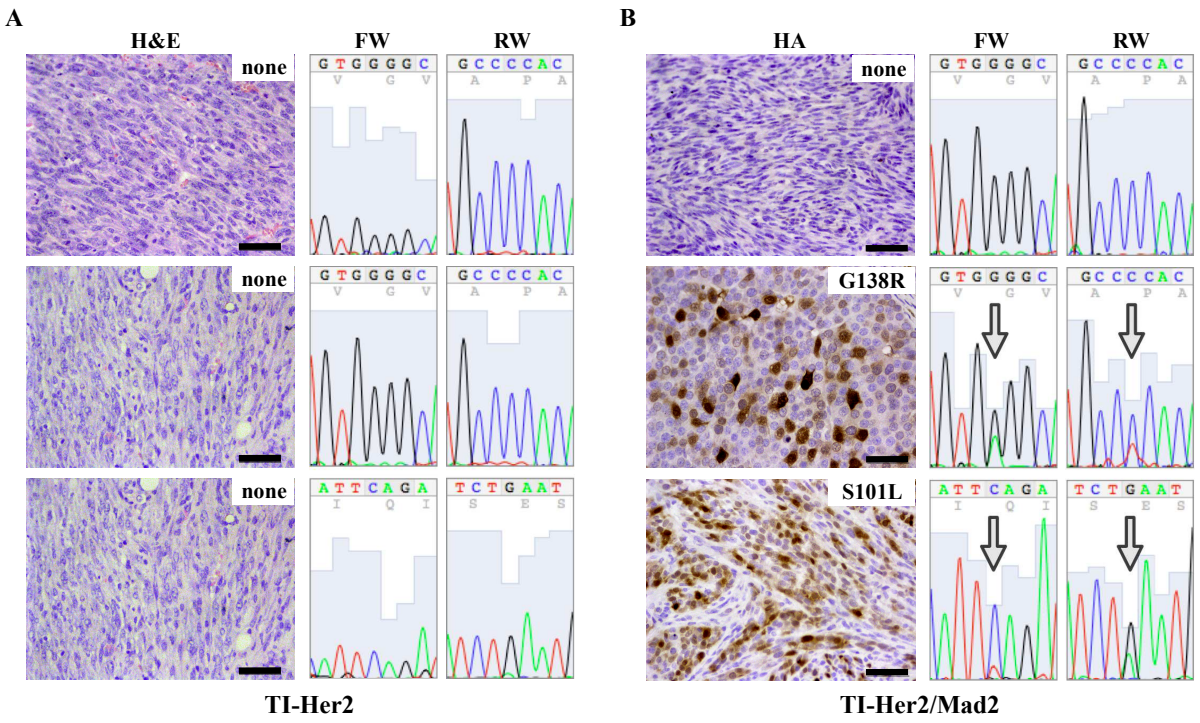

Figure 3.16: Mechanism of relapse in Her2 driven breast tumorigenesis. Histology examples (scale bar 50 µm) and relative analysis of rtTA sequencing region (FW and RW strand). A) TI-Her2 relapses have an EMT phenotype and do not mutate the rtTA region. B) TI-Her2/Mad2 relapses have either a solid or an EMT histology. HA IHC shows the Mad2 transgene re-expression, which is accompanied by mutation in the rtTA module.

3.5.4 Mad2 over-expression induces higher metastatic potential in solid tumor recurrences

It is known that human breast tumors preferentially metastasize to the bone, lung or brain (Kennecke et al., 2010). As mentioned in the introduction, CIN has been associated with poor patient's prognosis one of the parameters used to determine it, is the time required for the primary tumor to form distant metastasis.
TI-Her2 mice develop lung metastasis (Moody et al., 2002) which are responsive to doxycyline withdrawal. In mice, metastatic spread takes place preferentially through the blood vessels, while in humans, this happens mainly through the lymphatic system. This difference is intrinsic to the different organisms. Despite this, mouse models have proven useful in the metastasis field and can provide a mean to study this phenomenon in a complex organism.

Therefore, we sought to investigate the metastatic potential in case of Mad2 combination with the Her2 oncogene. To do so, we analyzed lungs from TI-Her2 and TI-Her2/Mad2 primary and recurrent tumors harvested at humane endpoint stage. In primary tumors, we did not find statistical difference the area of lung tissue occupied by metastasis among the two genotypes (Figure 3.17). Interestingly in some sporadic cases TI-Her2/Mad2 tumors metastasized very severely (up to 60% of metastatic lung tissue). It would be interesting to understand whether CIN influenced the metastatic potential: which was the aneuploid level in the primary tumor and if this was higher compared to less metastasis-prone ones. Nevertheless, the variability between triple transgenic mice is high and therefore, cannot account for a widespread phenomenon in the cohort. The conditions that rendered extremely serious lung colonization and, subsequently, macrometastasis formation have not been investigated

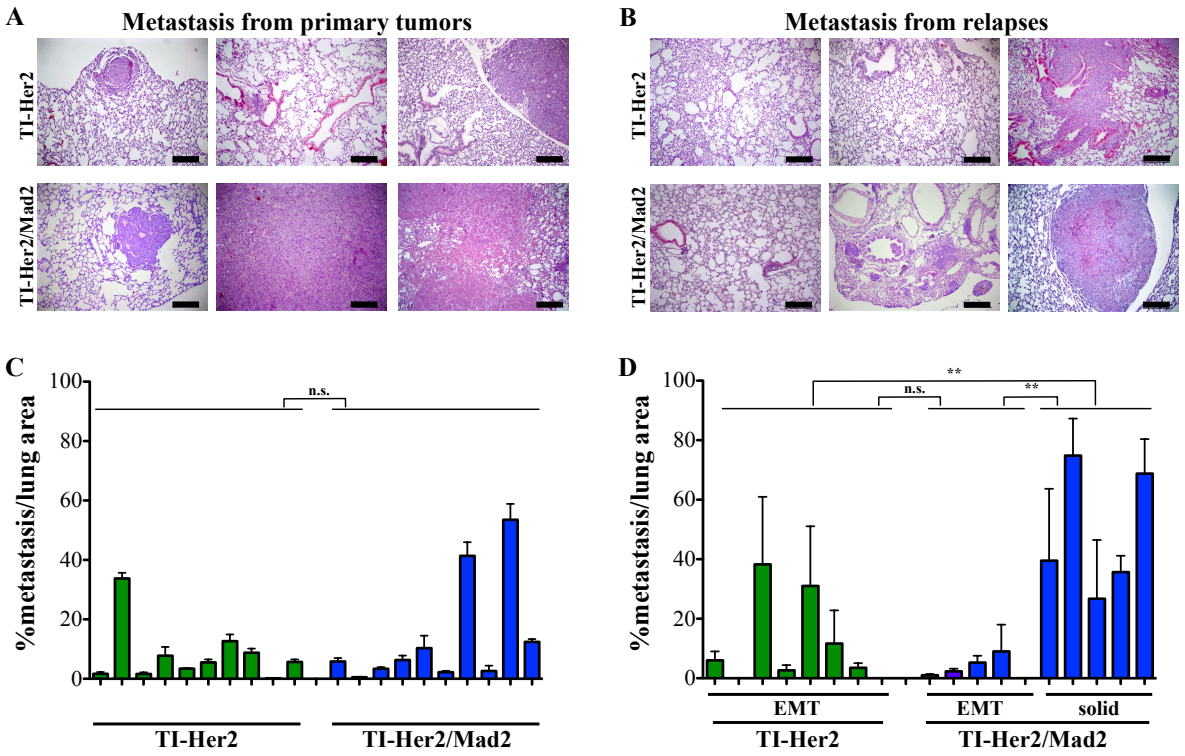

Figure 3.17: Mad2 over-expression promotes lung metastasis in TI-Her2/Mad2 solid recurrent tumors. A, B) Representative pictures of lung metastasis from primary A) or relapse tumors B) in TI-Her2 (upper panels) and TI-Her2/Mad2 mice (lower panels). Upper panels scale bar 200 µm. C) Analysis of metastatic potential - quantified as percentage of lung tissue area occupied by metastatic cells - in primary TI-Her2 (green) and TI-Her2/Mad2 (blue) lungs taken from mice bearing tumors at human endpoint. There is no statistical difference (unpaired t-test, p-value$>$0.05) between the average of metastatic potential in the two genotypes. D) Solid TI-Her2/Mad2 recurrent tumors have a higher metastastic potential, compared to TI-Her2 and EMT TI-Her2/Mad2 relapses (unpaired t-test).

yet. However it would be very interesting to understand whether there is a link between Mad2 induced genomic aberrations and this phenomenon.

In contrast to lung metastasis in primary tumors, in case of recurrent tumors, we made a distinction between TI-Her2/Mad2 solid and EMT-looking relapses. EMT recurrences show a low degree of lung metastasis, indistinguishable from the one of TI-Her2 relapses. In fact, it has been known for long time that murine EMT breast tumors do not form distant metastasis but preferentially invade local tissues (Cardiff R.D., 2010).
Solid TI-Her2/Mad2 recurrent tumors, instead, have a median percentage of metastatic lung tissue that is significantly higher of both EMT TI-Her2/Mad2 and TI-Her2 ones. Hence, in a specific subset of TI-Her/Mad2 relapses - which is representative of human relapse phenotype - Mad2 over-expression in the primary tumor created the conditions to promote the mutation of the rtTA element. This resulted, in turn, in transgenes re-activation and higher metastatic potential.

3.5.5 Mad2 induced CIN is not causative of higher DNA damage levels in primary tumors

Since solid TI-Her2/Mad2 relapses show transgenes re-expression and this is driven by a mutation in the rtTA transactivator, we sought to determine whether Mad2 over-expression

would induce higher levels of DNA damage in the primary tumors. Indeed, one could hypothesize that mutations should occur throughout the entire genome, however, given the system we are using, we are able to have a final readout monitoring transgenes de novo expression. Importantly, this renders tumors still addicted to the original oncogene, a phenomenon frequently observed also in human tumors (see chapter 1).
It has already been shown that CIN cells can have an increased mutational rate (Janssen and Medema, 2012). This can be due both to DNA double strand breaks and/or defective DNA repair. In particular, the Mad2 protein has been linked to the nucleotide excision DNA repair pathway (NER). Mad2 over-expression in cancer cell lines was shown to suppress phosphorylation of histone H2AX - a known marker of DNA damage - and to competitively interact with proteins mediating the NER response (Fung et al., 2008).

Hence, to test if Mad2 over-expression could lead to higher amount of DNA damage in our mouse model, we analyzed primary tumor samples with the Comet technique (for details see Materials and Methods). This assay allows the quantification of DNA damage in single cells, therefore, giving the possibility of discerning both the global amount of damage and its variability within the same tumor.
Six distinct TI-Her2 and TI-Her2/Mad2 primary tumors at humane endpoint stage were analyzed (Figure 3.18). In both genotypes, we could observe a quite spread heterogeneity in the amount and distribution of DNA damage. Comparison between the average of DNA damage within the same genotype, did not highlight any difference between TI-Her2 and TI-Her2/Mad2 samples (Unpaired t-test, p-value>0.05).
Therefore, Mad2 induced CIN, at least in this model of breast tumorigenesis, may not induce higher DNA damage. Alternatively, even if Mad2 might have provoked DNA damage, this result hinted at the fact that most of the lesions could be repaired to such an extent that rendered them not detectable with this technique.

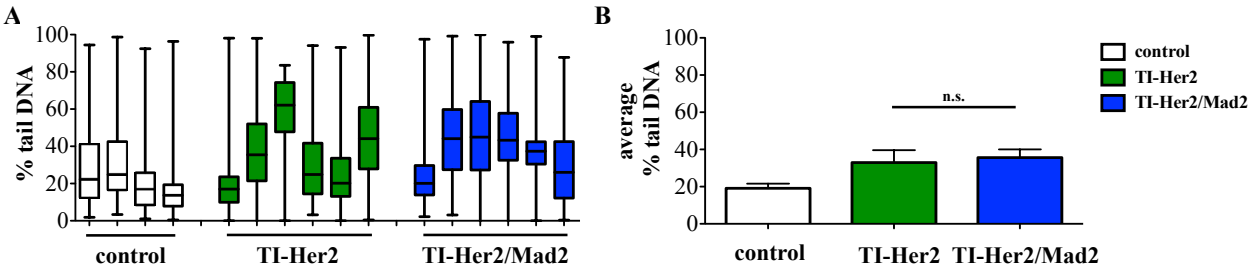

Figure 3.18: Mad2 over-expression did not induce higher levels of DNA damage in primary tumors. A) Box and whisker plot of the % of DNA in comet tails in individual samples; controls (white), TI-Her2 (green) and TI-Her2/Mad2 (blue) primary tumors. B) Average of DNA damage (% DNA in comet tail) within control, TI-Her2 and TI-Her2/Mad2 samples.

3.5.6 Study of primary tumors and relative relapses from the same animal

The analysis of primary and relapse tumor samples, had so far, been performed on malignant tissues taken from different animals. However, this way, it was not possible to study the evolution of a primary tumor into the corresponding relapse.
For this reason, we sought to investigate this question with two different approaches:

1. Biopsies on animals with primary tumors at human endpoint stage,

2. Injection of single cell suspension of primary tumors into Rag1$^{-/-}$ mice.

With these experiments, we aimed at understanding whether primary tumor histopathological phenotype could be predictive of relapse behavior. In particular, we wanted to understand whether the HA status in TI-Her2/Mad2 primary tumors might discriminate relapse outcome: would the absence of HA expression predict an EMT, TI-Her2 like type of relapse?

3.5.6.1 Analysis of biopsied primary tumors and corresponding relapses

Biopsies on animals with primary tumors at human endpoint stage (TI-Her2 n=11, TI-Her2/Mad2 n=21) were performed right before the moment they would be set off dox, then monitored exactly as non operated mice. Relapse latency did not show differences between non-operated and biopsied mice (Biopsied TI-Her2 mice 189 days, biopsied TI-Her2/Mad2 mice 107.5 days. Mantel-cox test, p-value>0.05 compared to corresponding non operated cohorts) (Figure 3.19). This suggested that, at least in these cohorts, biopsy did not affect the natural course of relapse in terms of latency. All TI-Her2 mice developed EMT relapses; while in the TI-Her2/Mad2 cohort, distribution of relapse phenotype among EMT (69%) and solid types (31%) was shifted towards TI-Her2 like. We cannot exclude the possibility that the biopsy procedure could have caused an inflammatory response that may have altered the relapse phenotype, skewing it to the EMT type.

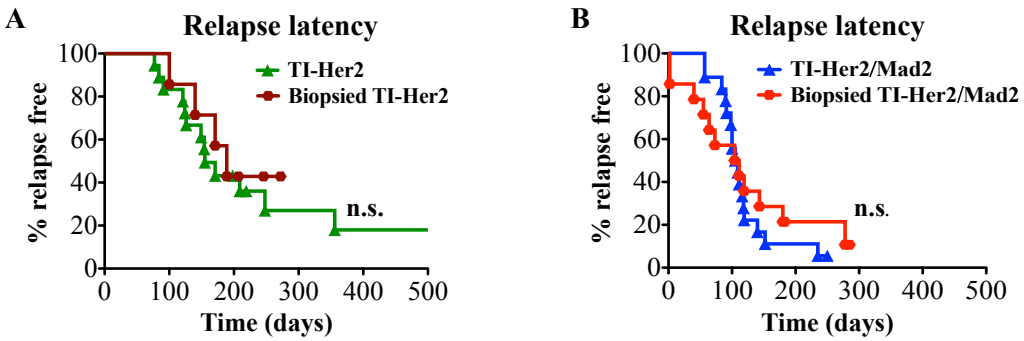

Figure 3.19: Biopsy procedure did not alter relapse latency. Relapse latency in biopsied TI-Her2 (A) and TI-Her2/Mad2 (B) mice did not differ from non operated mice (Mantel-Cox test, p-value>0.05).

Analysis of biopsied primary tumor samples from TI-Her2/Mad2 mice highlighted the presence of only one HA-negative primary tumor. Despite animals being selected for the longer primary tumor latency (see paragraph 2.4.1), we were not able to increase those numbers. Therefore, it was not possible to draw any conclusion in this respect. However, from results of HA-Mad2 positive biopsies we can infer that this type of primary tumor has the potential to relapse both as EMT (64% of cases) and solid (36%); therefore, EMT relapses may arise from both low and high Mad2 expressing cells. qPCR for HA-Mad2 and Her2 transgenes as well as rtTA mutation analysis gave similar results than non biopsied mice (Figure 3.20). This confirmed the ability of TI-Her2/Mad2 tumors to specifically mutate the rtTA region in order to sustain re-addiction to the Her2 oncogene.
Statistical analysis of Snail1 (unpaired t-test), instead, did not highlight any significant difference between solid TI-Her2/Mad2, EMT TI-Her2/Mad2 and EMT TI-Her2 relapses. This is in contrast to what we observed in non biopsied mice (see 3.5.3). In fact, solid and EMT TI-Her2/Mad2 relapses have comparable levels of Snail1 expression. It is known that an inflammatory response can stabilize the levels of Snail1 (Wu et al., 2009). Therof, even if we have not proven that the biopsy procedure was eliciting inflammatory responses, this

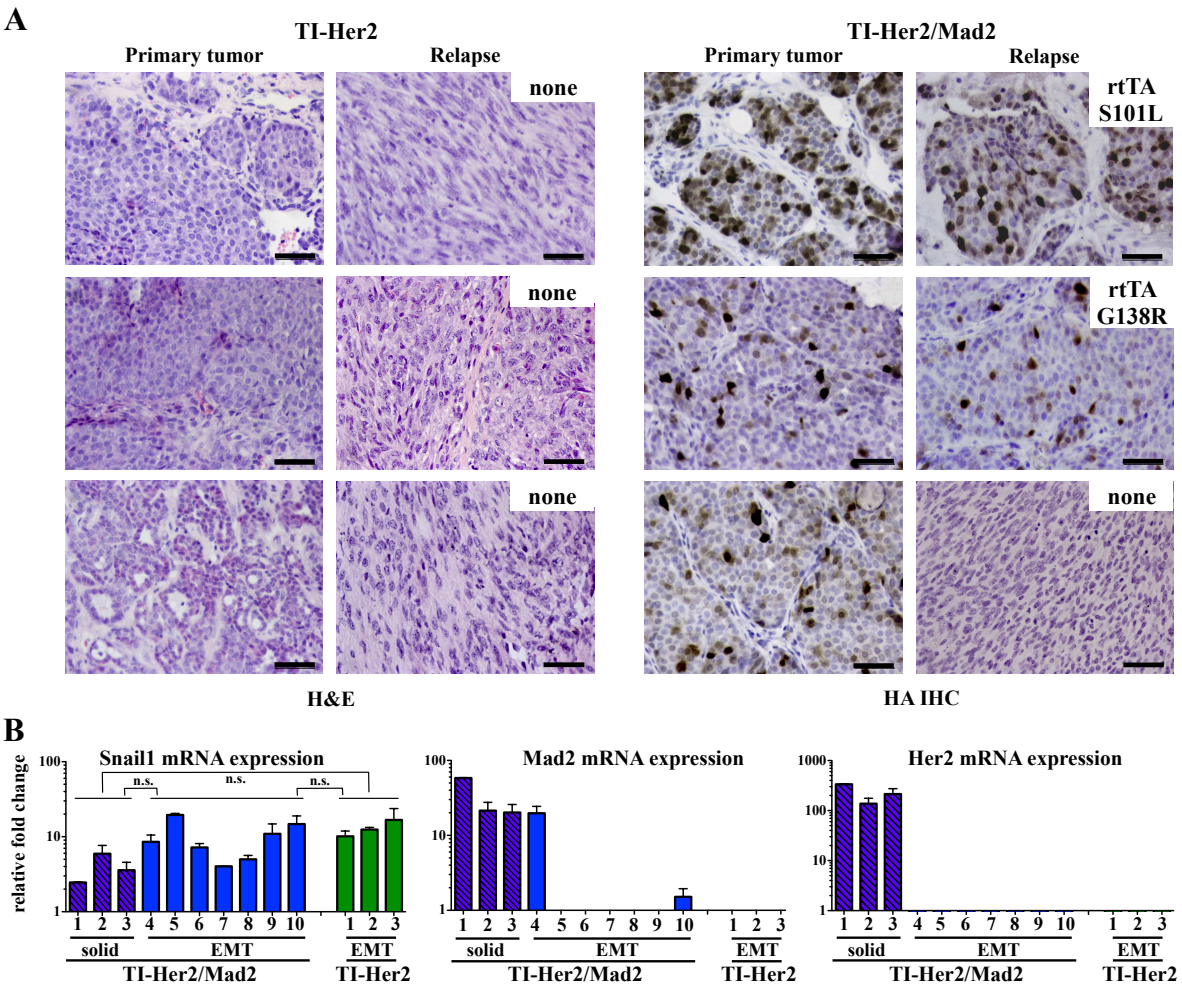

Figure 3.20: HA status in primary tumors did not predict relapse outcome in TI-Her2/Mad2 mice. A) Histopathological analysis of primary biopsies and corresponding relapses in TI-Her2 (H&E staining) and TI-Her2/Mad2 (HA IHC) samples. Panels in the top right corner of solid TI-Her2/Mad2 relapses describe mutational analysis of the rtTA region. All the other samples do not bear any mutation. (Scale bar 50 µm). B) qPCR analysis of Snail1 and transgenes expression. TI-Her2 relapses (green), EMT-like TI-Her2/Mad2 (plain blue) and solid TI-Her2/Mad2 recurrences (striped blue).

might provide a possible explanation for Snail1 levels becoming homogeneous across all our samples.

3.5.6.2 Analysis of primary tumors injected into Rag1$^{-/-}$ mice

As mentioned in the previous paragraph, the biopsy procedure itself could generate physiological responses that may change the outcome of relapse. An extreme advantage of the mammary gland is that it is a transplantable organ. Therefore, we decided to inject single cell suspension of primary tumor into the mammary gland of Rag1$^{-/-}$ immuno-compromised mice. At weaning, the development of the mouse mammary gland is not completed yet, and the mammary tree is still not branched. A procedure of clearance of the mammary tree originating center leaves the mammary fat pad intact and this can be used to inject cells into a physiological environment.
Primary tumors from TI-Her2 (n=17) and TI-Her2/Mad2 mice (n=14) were digested into single cell suspension (see Materials and Methods) then injected into cleared mammary fat pad of 21 days old Rag1$^{-/-}$ hosts (number of injected cells 250,000 or 500,000 per fat pad).

After, Rag1$^{-/-}$ mice were monitored under two conditions: in one case, mice were fed with normal food, thus mimicking oncogenic shock; in the other, mice were kept on a doxycycline diet until injected cells could grow again into a solid palpable mass and, only then, set back to a normal diet to switch off transgene expression.
Of all TI-Her/Mad2 tumors injected, only two of them managed to grow into a solid mass when the mouse was put off dox straight after the injection; only one analogous case appeared in the TI-Her2 cohort. This condition, apparently does not allow tumor cells to find an environment amenable to relapse.
The outcome was different in the other condition: when mice were set on a doxycycline enriched diet, in most of the cases a tumoral mass would form with quite short latency (2 to 4 weeks after injection). After doxycycline withdrawal, tumor generally regressed, even if some never reached the non-palpable state. This discrepancy could be due to the mammary gland sparing procedure itself: as cells were injected when the mammary gland is not formed, the draining system of the organ never developed, therefore making more difficult the elimination of dead tissue. Eventually, completely regressed tumors grew into relapses or non completely regressed ones resumed growth.

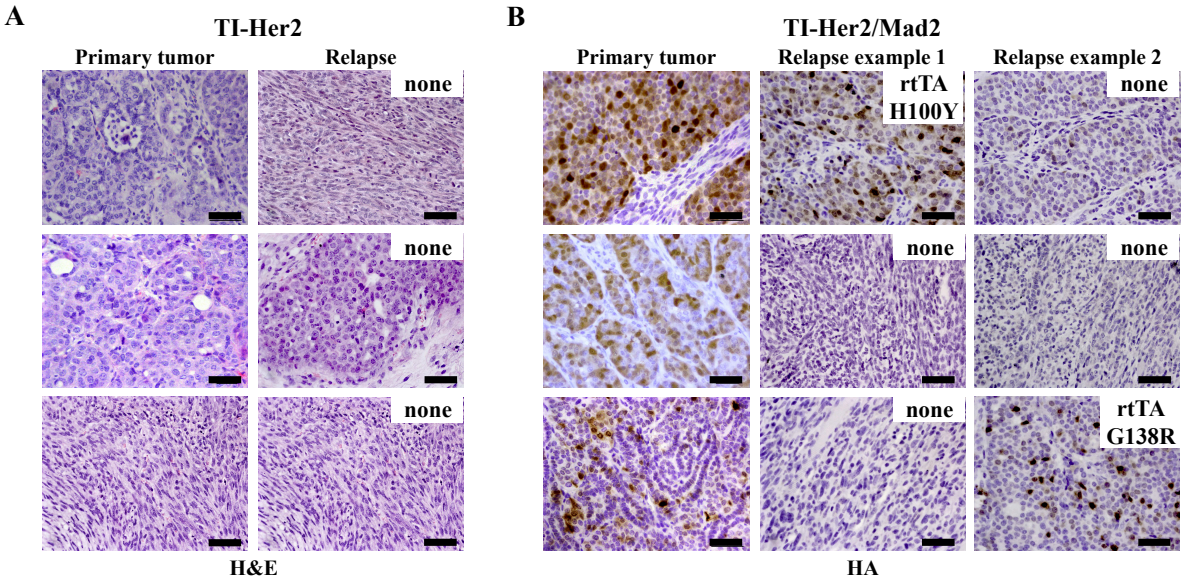

Figure 3.21: Analysis of TI-Her2 and TI-Her2/Mad2 relapses in Rag1$^{-/-}$ mice. A) Primary tumors and corresponding relapses from TI-Her2 cohort (H&E). In the middle panel, an example of solid relapse is shown. B) HA IHC of TI-Her2/Mad2 primary tumors and corresponding relapses. Insets describe, if present, the type of rtTA mutation. Scale bar 50 µm.

In agreement with biopsied mice, relapses arising into Rag1$^{-/-}$ recapitulated what happens with non-operated animals in TI-Her2/Mad2 cohort. Primary tumors positive for HA-Mad2 IHC had the potential to relapse both as EMT and solid way, and the latter ones had a mutation in the rtTA region that would allow transgenes re-expression. However, the striking finding was that the very same pool of injected cells coming from the same primary tumor could give rise to both types of relapse. What is the event leading to a specific "choice" towards relapse is not clear; when this decision is made is also an interesting point to be investigated.

Regarding TI-Her2 mice, tumors relapsed via an EMT phenotype. In this case, for the first time, two solid relapses arised but they did not present an rtTA mutation. None of the relapses coming from TI-Her2 primary tumors beared any mutation.

Conclusively, these results are confirming what we have already observed in biopsied and non biopsied mice in terms of relapse. We were able to show that:

- in non biopsied mice, TI-Her2 primary tumors relapse with an EMT phenotype via the up-regulation of the Snail1 transcription factor, as previously published.
- TI-Her2/Mad2 primary tumors give rise to relapses that can have either an EMT or solid phenotype. In the first case, recurrent tumors have the same characteristics as TI-Her2 derived ones. When a solid relapse occurs, mutation on the rtTA element promotes transgenes re-expression. This highlights the dependency of primary tumors on the oncogenic initiating lesion. Therefore, in our transgenic system, mutation of the rtTA element is a preferred hijack to sustain tumor growth.

Chapter 4

Discussion

CIN has been observed in a wide variety of human tumors (Duijf et al. 2012). A recent study has pointed out that 68% of human solid tumors show unstable aneuploidy. The rates of whole chromosome aneuploidy vary among tumor types, with brain tumors being characterized by the highest incidence, most probably because samples are amenable to analysis only at the very late stages of the disease. In the case of breast tumors, more than 60% of analyzed samples are aneuploid.
Trying to understand the correlation between aneuploidy, CIN and tumorigenesis is extremely important. In the case of breast cancer, it represents the second leading cause of cancer-related death in women worldwide. If prevention, early diagnosis and monitoring of familial cases have improved the timing of neoplasia detection at early *in situ* stages, much more still has to be done regarding therapeutical options and, in particular, recurrence.

Mitotic checkpoint genes are frequently over-expressed in human tumors (Carter et al., 2006; Rhodes et al., 2004; Rhodes et al., 2007) and it has been extensively demonstrated that deregulation in their expression is one of the molecular mechanisms generating CIN *in vivo* (Diaz-Rodríguez et al., 2008; Schvartzman et al., 2011; Sotillo et al., 2007). In human tumors, over-activation of the SAC is an event that happens much more frequently than mutation (Perez de Castro et al., 2007) or partial inactivation. In breast tumors, CIN scores are highly associated with patients poor prognosis (Carter al., 2006; Smid et al., 2010) and are thought to favor the generation of tumor heterogeneity and acquisition of resistance to therapeutical treatments.

In this dissertation, we provide, for the first time, evidence for Mad2 driven CIN to play an opposing role in breast tumorigenesis, in the context of both Her2 and c-MYC oncogenic backgrounds. Mad2 over-expression, and subsequent over-activation of the SAC in the process of tumor formation, acts as tumor suppressing event and delays breast tumorigenesis. After mimicking perfect targeted therapy, exogenous Mad2 over-expression in primary malignancies is able to promote tumor relapse, thereof mirroring what happens in human tumors.

In this chapter, I will discuss the results previously presented and highlight the unanswered questions arising from this work.

4.1 Mouse modeling of human breast cancer

Mouse models that would recapitulate CIN in an oncogenic background are still scarce (Janssen and Medema, 2012) and no model has, so far, been published for breast cancer.

Therefore, we thought that generating a new mouse model, that would faithfully recapitulate breast tumorigenesis, will be of extreme help in the understanding of this disease.
Unlike previously published results (Sotillo et al., 2007), Mad2 over-expression alone is not sufficient to initiate primary tumor formation in the breast, at least in the MMTV-rtTA background. Therefore, we crossed tetracycline inducible mice, overexpressing the Mad2 protein in combination with either the Her2 or c-MYC oncogenes specifically in the mammary gland. As previously mentioned, these are relevant oncogenes in human breast cancers. Whereas MYC is still not druggable, targeted therapy has been developed against the Her2+ subtype. Nevertheless, despite initial response to trastuzumab treatment, a high percentage of patients eventually relapse becoming resistant to this antibody.
The mouse models we generated (TI-Her2/Mad2 and TI-MYC/Mad2) allowed us to study breast tumorigenesis combining both CIN and the concept of oncogene dependence in the breast. All the steps, from primary tumor formation, progression and relapse could be studied. In particular, these mouse models exactly recapitulate what happens in humans after targeted therapy treatment - in a realistic scenario for Her2, while potentially predicting targeted therapy outcome against the c-MYC oncogene. In both cases, Mad2 induced CIN renders ineffective the initial treatment, making these mice a precious tool amenable to study molecular mechanisms at the source of resistance.

The Her2 oncogene over-expressed in our model was cloned from rat and, unfortunately, is not recognized by the human monoclonal antibody trastuzumab. However, these mice could be alternatively treated with other drugs that have been developed to overcome trastuzumab resistance. These are small molecules inhibitors (such as Neratinib or Laptinib) that are able to recognize and bind also to rat Her2 and could be given to mice in appropriate regimens. This would have the advantage of uncoupling Mad2 over-expression from oncogene withdrawal, allowing the persistence of CIN during therapeutic treatment. It would be interesting to understand whether TI-Her2/Mad2 mice would develop relapses with the same or an even shorter latency in this setting. In the first scenario, this would lead to the hypothesis that oncogene independent clones are generated already in the primary tumor, therefore, the persistence of CIN would not confer any advantage in tumor recurrence. On the other hand, if relapses occured faster, it would mean that the acquisition of further rearrangements and/or mutations are required to promote tumor re-growth and that CIN has the capability of fueling this process.

As far as modeling of the human disease *per se* is concerned, the simultaneous regulation of both Mad2 and Her2/c-MYC by doxycycline administration/withdrawal is a feature that leaves several questions open. For instance: when, during primary tumorigenesis, is CIN crucial to promote selection of oncogene independent clones? And how would it be possible to better mimic targeted therapy while still giving CIN the possibility to act on cancer evolution towards resistance?
These questions obviously cannot be answered with the TI-Her2/Mad2 and TI-MYC/Mad2 models since Mad2 over-expression occurs at the same time as the oncogenes. In primary tumor formation, this means that CIN will be induced since the very early stages. If it is true that aneuploidy is one of the earliest marks of the disease, which can be detected also in histologically normal breast tissue, we still do not know if this happens concomitantly with oncogene deregulation. Since oncogene over-expression has always been depicted as the driving force of tumor initiation, it has been hypothesized that one event follows the other. The advent of next generation sequencing has given the opportunity to deeply investigate this question and now we start having first insights from human tumor specimen. In a paper recently published by Wang and colleagues (Wang et al., 2014) sequencing studies performed

on two different subtypes of human breast tumors revealed that chromosomal rearrangements defined a monoclonal population, therefore occurred early in tumor evolution. On the other hand, the mutational landscape evolved gradually generating widespread clonal diversity. Unfortunately, it is not clear whether aneuploidy could have evolved with co-existing mutations. Hence, if it was clear that two different molecular mechanisms operated in the same tumor, what is still obscure is the sequence of events during tumor evolution. In this regard, it should be pointed out that all the information we have acquired come from biopsy or mastectomy specimen, which represent later stages in the progression of the disease and force retrospective types of studies.

Then, when CIN is an established feature of neoplastic lesions, what is the time frame during which aneuploidy needs to act in order to fuel the selection of therapy resistant clones? The only way to answer this question lays in the generation of an improved mouse model in which Mad2 and oncogene over-expression are uncoupled. Therefore, in our lab, new mouse models - for breast and lung tumorigenesis (M. Jechlinger and C. Aguirre unpublished results) - have been generated. In these mice Mad2 is under doxycycline regulation, while oncogenes are controlled by Cre recombinase induction. This way, Mad2 expression can be independently turned on, at later stages, at different timepoints and for different timeframes of induction. Tamoxifen administration will induce the Cre-driven excision of a stop cassette cloned upstream the oncogene, thus allowing its constitutive expression without interfering with Mad2 induction. In the same way, doxycycline withdrawal will not affect oncogene over-expression and, conversely, administration of targeted therapy to mice would not simultaneously shut off Mad2 induced CIN. In fact, it is likely that in human patients CIN is still an active process during treatment. Therefore, in this case, another possible scenario of the disease could be modeled and investigated.

4.2 Tumor suppressing effects of CIN

As extensively discussed in the first chapter of this dissertation, an increasing body of evidence has pointed out the opposing roles of CIN in tumorigenesis: tumor suppressing and tumor promoting. The tumor suppressive role of CIN has been demonstrated both in yeast (Torres et al., 2007) and *in vivo* mouse models (Rao et al, 2005). In particular, Rao et al. were able to demonstrate the dual role of CIN in the same animal. In fact haploinsufficiency of BubR1 decreased the rate of small intestinal tumors and at the same time promoted colon tumorigenesis in the $APC^{Min/+}$.
In a recent mouse model of BubR1 over-expression, mice were protected against tumor formation even in an oncogenic background (Baker et al., 2012). Both cyclin B1 and B2 over-expression, instead, promoted spontaneous and carcinogen induced tumor incidence (Nam and van Deursen, 2014).

In the mouse models generated in this dissertation, Mad2 induced CIN has a clear tumor suppressive effect in primary tumor formation both in the Her2 and c-MYC backgrounds. Unpublished data from our lab, using a mouse model where Mad2 is expressed in combination with another oncogene, $Kras^{G12D}$, led to the same observation (K. Rowald, in preparation). Moreover, tumor multiplicity was reduced in TI-Her2/Mad2 in comparison to TI-Her2 animals. These results highlight the hypothesis that Mad2 detrimental role on primary breast tumorigenesis may be a general effect, which is independent of the oncogenic background but linked to tissue specific properties of the mammary gland. In fact, in contrast with this finding, no difference in tumor multiplicity - but acceleration in tumor formation - was observed in a CIN mouse model of $Kras^{G12D}$ lung adenocarcinoma, as compared to $Kras^{G12D}$

mice alone (Sotillo et al., 2010).

In section 2.3, Mad2 over-expression was demonstrated to cause mitotic arrest *ex vivo* after a short pulse of induction. This result was further confirmed *in vivo* (K.Rowald). If these cells are arrested in mitosis, they will eventually overcome the block and progress through cell division. Furthermore, these cells will have to adapt to Mad2 over-expression in order to overcome CIN detrimental effects. As already discussed, the outcome of incorrect mitosis generates aneuploid cells whose fate depends on the cell type, the extend of genomic imbalance and DNA damage that has been generated. Aneuploid cells constitute a danger for a normal organism, therefore, when the main tumor suppressive pathways are active, they are detected and driven into apoptosis or senescence. However, in a tumorigenic background, such as the one generated by oncogene over-expression, already transformed cells will not readily be eliminated when becoming aneuploid and will, eventually, adapt to Mad2 over-expression.
Live cell imaging of pre-neoplastic lesions of the mammary gland are still technically very challenging. Therefore, transgenic primary mammary epithelial cells grown in 3D could be used as a valid alternative to monitor this phenomenon. In fact, these cells are responsive to doxycycline induction and can be monitored in time-lapse experiments to understand the outcome of cell division upon Mad2 over-expression. First cell divisions will give a hint of the consequences of prolonged mitosis in primary mammary cells, while second or third rounds may help understanding how, and if, cells can overcome mitotic block. For instance, time required to complete mitosis may change and allow cells to more readily slip through the block imparted by Mad2 over-expression.
For this reason, we crossed mice constitutively expressing the H2B-GFP protein with our bi-transgenic and tri-transgenic mice to perform live cell imaging of 3D cultured cells. This experiment has proven to be very challenging from the technical point of view, therefore results are not presented in this dissertation. However, this may be one of the most straightforward ways to understand the mechanism of cell adaptation to Mad2 induced mitotic block and CIN.

It has been demonstrated that Mad2 over-expression is not required for tumor maintenance (Sotillo et al., 2007). This directly correlates with the observation that a subset of TI-Her2/Mad2 primary tumors do not show Mad2 over-expression at human endpoint stage. We could hypothesize that Mad2 expression was cell autonomously down-regulated, therefore, eliminating detrimental effects of continuous generation of genetic imbalances once an aneuploid karyotype, favorable for tumorigenesis, has been selected. In fact preliminary results coming from the analysis of transgenes expression in induced mammary glands and primary tumors taken from the same animal at humane endpoint stage, highlighted the loss of Mad2 expression only in breast cancers (see 3.2.4.1). Hence, we could speculate that after doxycycline induction all mammary glands expressed both transgenes, but the selection for HA-Mad2 negative clones took place only during tumor formation. Of course, this is only an indirect correlation and the best way to demonstrate this hypothesis would be the comparison between initial and humane endpoint levels of induction. Therefore, analysis of transgenes expression at early pre-neoplastic time points throughout all primary tumor formation has now been undertaken in the lab (K. Rowald, unpublished results). These data hint at the hypothesis that whenever Mad2 is over-expressed at high levels cells down-regulate it to reach average amount of protein. As consequence, this may lead to tolerable CIN levels that are not detrimental to cellular fitness but promoting tumor heterogeneity and the selection of therapy resistant clones.
The Weaver lab (Silk et al., 2013) has respectively correlated mild and high aneuploidy levels

with tumor promoting and tumor suppressing effects *in vivo*. One backdraw of this study is that levels of aneuploidy were scored in MEFs and not in cells taken from primary tumors. Still, both *in vitro* and *in vivo* data elegantly supported a dual role for CIN in tumorigenesis (see 1.3.3).
Nevertheless, the mouse models generated in our lab may underlie a similar phenomenon, whereby initial high CIN levels are detrimental for tumor formation and therefore, selected against. After cell autonomous down-regulation of Mad2 expression or selection for cell expressing an intermediate level, evolution of tolerated tumor promoting karyotypes can take place. The initial detrimental effect of Mad2 over-expression may explain delayed primary tumor latency in TI-Her2/Mad2 and TI-MYC/Mad2 mice, while the consequent level adaptation will allow formation of aneuploid tumors.

4.3 CIN and the generation of tumor heterogeneity

Aneuploidy has long been known to promote tumor heterogeneity. This characteristic is fundamental to drive the acquisition of therapy resistant karyotypes that would, ultimately, lead to ineffective therapeutic approaches.

In case of MYC driven tumorigenesis, there was no difference in the histopathological spectrum of double and triple transgenic mice both in primary and recurrent tumors. Why Mad2 over-expression was not broadening this phenotype is not clear. As already mentioned (see 1.4.3; Cardiff et al., 2000) GEM tumorigenesis driven by specific transgenes is characterized by unique histologies. Thereby, one could speculate that in this case, the MYC oncogene is the principal determinant of the phenotype and Mad2 over-expression cannot over-ride this "specification".
In contrast, in the TI-Her2/Mad2 mouse model we could appreciate a higher heterogeneity in tumor histopathology, both in primary cancers and relapses when compared with the TI-Her2 counterparts. In tumor recurrences, solid histopathology was found only in TI-Her2/Mad2 samples. Human breast cancer relapses are characterized by solid phenotype only. Therefore, at least in a subset of TI-Her2/Mad2 relapses we were able to recapitulate the human phenotype. Moreover, these were the only ones that showed re-expression of the initiating oncogene, a mechanism of relapse that is common in human patients (Berns et al., 2007; Christianson et al., 1998; Garner et al., 2013).
To investigate whether a histological broader phenotype in TI-Her2/Mad2 mice could be due to higher genomic aberrations, we analyzed TI-Her2 and TI-Her2/Mad2 primary tumor karyotypes taking advantage of the M-FISH technique.

Mad2 over-expression in MEFs under the control of the CMV (citomegalovirus) promoter (Sotillo et al., 2007) is known to induce anaphase bridges, chromosome gains and losses, chromosome breaks and rearrangements.
Karyotype analysis (Montagna et al., 2002) of Her2/neu induced mammary gland tumors, in a mouse model where an activated form of the Her2 transgene is expressed under the control of its own endougenous promoter, highlighted recurrent deletions of chromosome 4 and genomic amplifications. Therefore, we wanted to understand whether Mad2 over-expression in the Her2 oncogenic background would promote higher rates of genomic instability compared to TI-Her2 primary tumors. In conclusion, TI-Her2/Mad2 primary tumors are more aneuploid and this correlates with more cells missegregating chromosomes in mitosis and W-CIN.
Suprisingly, there is no statistical difference in the amount of S-CIN between the two genotypes. As Mad2 over-expression has been shown to promote the formation anaphase bridges

and chromosome breaks, it was likely to expect that triple transgenic tumors would be distinguished by a higher rate of rearrangements, detected by M-FISH as translocations or derivative chromosomes. Why this previous observation has not been found in our model could be due to different reasons. First, TI-Her2 tumors are already *per se* characterized by a certain level of chromosome rearrangements and Mad2 over-expression may not affect this parameter, therefore no difference is observed. Second, primary tumor samples were collected at human endpoint stage. As discussed in the previous section, tumors need to select for an optimum level of CIN in order to maintain heterogeneity compatible with cellular viability. Therefore, it might be possible that in breast tumors, once a certain level of CIN has been reached, it cannot be modified to avoid effects compromising cellular fitness. Probably, analysis at earlier time points may reveal pre-existing differences that are not maintained in established primary tumors.

One open question is the identification of those genomic aberrant clones that will give rise to tumor recurrence. Karyotyping analysis of relapses may highlight the existence of recurrent chromosome gains/losses or rearrangements which could be analogous to a subset found in primary tumors.
Alternatively, we decided to take advantage of 3D cell culture system to grow primary tumor cells into an organotypic environment. The possibility of withdrawing doxycycline from the culture medium gave us the possibility to mimic oncogenic shock and select for clones that would grow independently, despite silencing of the oncogene. All cells that had not already acquired this characteristic would be eliminated by cell death, while allowing the expansion of clones with the potential of generating a relapse. This selection method has proved harsh on primary tumor cells. We faced problems in the growth and propagation of oncogene independent clones. This could be due to the technical procedure itself but may also hint at the fact that additional mutations are required to promote tumor relapse and, therefore, the regressed state should be mimicked in culture as well. In fact the mammary gland is not a resting tissue: female mice are constantly exposed to hormones that regulate the reproductive cycle from puberty. This results in a periodical expansion of different cell populations, including stem cells. Therefore, an expanding and cycling population could represent a putative target for cell transformation events at specific windows of the adult reproductive cycle (Joshi et al., 2010). This could mean that a static 3D culture system is not enough to mimic what happens *in vivo*. As cells should be cycling, subsequent re-seeding of the 3D gels would force these cells into proliferation and could create the conditions to acquire mutations.

4.4 Mad2 induced CIN promotes acquired resistance to targeted therapy

The concept of oncogene dependence (Weinstein and Joe, 2006) describes the addiction of cancer cells to the sustained expression of the driving oncogenic lesion both for the initiation and the maintenance of the malignant phenotype. Demonstrations of this phenomenon came from studies on cell lines and mouse models, thus opening the path for the advent of modern targeted therapy. The past decade has seen the development of many drugs, specific inhibitors for over-expressed tyrosine kinase receptors which are currently widely used in the clinics. For example, Imatinib to cure gastrointestinal stromal tumors and certain types of leukemias, erlotinib and gefitinib targeting EGFR in non small cell lung cancer, sutinib against VEGF receptors in kidney malignancies and trastuzumab, the already mentioned monoclonal antibody against the Her2 receptor. All these agents have brought tremendous

advance in the treatment of cancer disease. Most of them ensure better quality of life during treatment while others, such as trastuzumab, can also improve overall patient survival and time to develop recurrence.
However, as already mentioned, those therapies may not be effective for two reasons. First, the patient is intrinsically resistant to the drug, therefore there will not be any benefit during its regimen. Second, tumors develop secondary resistance to the drug, eliciting mechanisms of adaptation and becoming refractory to its function. This leads to the formation of tumor recurrences which do not respond any more to the initial treatment and, therefore, represent a major problem in the clinic.

Then, it is of no surprise that a huge effort has been put to understand molecular mechanisms underlying resistance. This is currently one of the most widely investigated question in the cancer field. CIN has been associated with poor patient outcome and short disease free survival for long time. This observation generated the concept of unstable aneuploidy as a driving force for tumor heterogeneity and selection of therapy resistant clones.
The first mouse model that faithfully recapitulated and demonstrated that CIN is one of the molecular mechanisms favoring tumor recurrence was published by the Benezra lab (Sotillo et al., 2010). This phenomenon was modeled generating a mouse over-expressing in a conditional manner Mad2 and $Kras^{G12D}$ oncogene specifically in the lung. Lung adenocarcinomas develop only as a consequence of $Kras^{G12D}$ expression and, when in combination with Mad2, these tumors show a high degree of aneuploidy. As demonstrated by MRI monitoring, both $Kras^{G12D}$ and $Kras^{G12D}$/Mad2 primary tumors regress upon oncogene withdrawal. Remarkably, primary tumors driven by $Kras^{G12D}$ alone never developed recurrence. In striking contrast, when Mad2 was over-expressed in the primary tumor, 50% of mice relapse, highlighting CIN as a potential mechanism to drive the generation of Kras independent clones. Relapse analysis pointed out a variety of differentially activated pathways, confirming the advantage of CIN tumors to select many diverse roads to escape treatment.

Thus, the results presented in this dissertation further confirm this hypothesis, for the first time in a CIN mouse model of breast cancer. In fact, both in TI-Her2/Mad2 and TI-MYC/Mad2 mice tumor recurrences arose with significantly shorter latencies compared to the double transgenic counterpart. In the case of Her2 driven tumorigenesis, these results were confirmed also in biopsied mice and transplanted tumor into immune-compromised hosts.
The mechanisms of relapse were heterogeneous within the same cohort of mice, stressing again the notion that aneuploidy gives the possibility of choosing alternative pathways to overcome oncogene withdrawal. As in human patients, in some cases, mutations that alternatively re-activate the same oncogenic arm would occur. This observation correlates not only in Her2+ trastuzumab treated breast cancer patients, but also with EGFR erlotinitib treated lung cancers. In the last case, a point mutation disabling drug efficacy occurs in the very same receptor, thus keeping the pathways activated even in the presence of the drug (Kobayashi et al., 2005; Pao et al., 2005; Politi et al., 2010).

It is widely known that cancer cells have impaired DNA repair pathways (Hanahan and Weinberg, 2000), thereby providing a fertile soil for the accumulation of mutations. On top of it, also CIN may help the disruption of DNA repair pathways (Janssen and Medema, 2012). The observation that triple transgenic mice in our cohorts relapse via specific point mutations (see 3.4.2 and 3.5.3) lead us to the hypothesis that Mad2 over-expression might favor this process (Fung et al., 2008). Nevertheless, we could not demonstrate that mice bearing Mad2 over-expression in Her2 driven primary tumors have higher amount of DNA

damage (see 3.5.5). The technique we used (see 2.3.3) is a sensitive method to measure at single cell level single and double strand breaks in DNA. The fact that we could not appreciate stastistical difference between the average amount of DNA damage in TI-Her2 and TI-Her2/Mad2 samples might be explained in different ways. First, Mad2 over-expression does not induce higher DNA damage. Second, even if Mad2 induced CIN caused DNA damage, this was repaired and, therefore, not detected. Both points may also correlate with the fact that TI-Her2/Mad2 primary tumors are not more frequently rearranged than their double transgenic counterpart (see 3.2.4.2 and 4.3). Third, a different technique, such as sequencing, might be more suitable to adress this question and unravel the presence of different mutational rates between the two genotypes.

Nevertheless, it should be kept in mind that aneuploidy is a valid route for adaptation to multiple rounds of therapy: it is a constant fuel for the generation of new genomic imbalances that will mine the efficacy of subsequent and/or combinatorial agents.

4.5 The SAC as a therapeutic target

The mitotic checkpoint is an essential machinery in mammals, both in normal and transformed cells. The idea of the MC as a druggable target has for long been exploited in the treatment of human tumors. Adjuvant chemotherapeutic treatments against the SAC are often used in combination with conventional targeted therapy. These drugs target microtubule polymerization, either stabilizing microtubule dynamics (for instance, taxol) or promoting their depolimerization (i.e. vincristine and vinblastine). Microtubule targeting agents have considerable toxic effects, which range from neurotoxicity, thrombocytopenia, bone marrow suppression to reversible hair loss. This is, of course, a consequence of targeting such a ubiquitous protein like tubulin. Patients may eventually develop resistance, usually due to point mutations in the drug binding site of the tubulin monomer (Wang et al., 2005). All these agents are thought to act on the SAC by perturbing the mitotic spindle and promoting arrest at the metaphase plate. These results came from *in vitro* studies of cultured cells (Jordan et al., 1993). Mitotic block is not a condition that can be indefinitely maintained and, eventually, cells will slip through. Consequences of mitotic block depend on the cell type and may vary even among the cells belonging to the same organ (Das et al., 2001). After prolonged mitotic block, cells may die during mitosis, slip through and subsequently undergo apoptosis during interphase or progress in the cell cycle to different rounds of mitosis. Aneuploid cells may anyhow not be eliminated, especially in the fertile soil of mutated genetic background of pre-neoplastic lesions. Therefore, if, on one hand, microtubule targeting drugs usually have strong therapeutic effect at the beginning of the treatment, on the other, they may also promote tumor progression through the selection of karyotypically unstable clones.
Contrariwise an increased mitotic index does not seem to be a requirement - at least for taxol efficacy - at the concentration measured *in vivo*. Zasadil and colleagues (Zasadil et al., 2014) demonstrated that tumor cells taken from breast cancer patients, who received taxol treatment, do not necessarily undergo mitotic arrest. Tumor cell death was caused by the formation of multipolar spindles, which, in turn, resulted in chromosome missegregation and generation of aneuploid cells.

Moreover, the concept of different levels of aneuploidy as tumor promoting or suppressive should be carefully taken into account. As quite promoted therapeutic approach, it must be kept in mind that it still has obscure dynamics and should be further investigated.
We know that high levels of CIN are usually not well tolerated and induce higher rates of cell death (Silk et al., 2013) but what this exactly means in terms of actual frequency of

missegregation events is not clear. We still do not know which is the threshold of instability that once overcome will inevitably induce cell elimination. Furthermore, it should be considered that different cell types may be sensitive to different CIN levels, so each case should be treated differently. Then, how this would practically be achieved in patients is yet not clear. The right drug concentration should be specifically and equally delivered only to the tumor mass to avoid off target effects and dose dependent responses.

Cancer cells are usually characterized by an over-activation of the mitotic checkpoint. Therefore, if a concept analogous to oncogene-dependence should be exploited, then inhibition of the mitotic checkpoint may have therapeutic results as well. This approach gave promising results in glioblastoma cultured cells (Tannous et al., 2013). When the Mps1 mitotic kinase was inhibited, cells became more sensitive to vincristine treatment. The combination of these two agents promoted mitotic over-ride, increased aneuploidy and enhanced cell death. Even though these results are promising, it is still not clear how targeting of tumor cells only could be achieved in patients. Nevertheless, these findings open new possibilities in the treatment of CIN tumors.

We still do not completely understand the molecular mechanisms underlying the relationship between tumor biology, CIN and therapy resistance. Great improvements have been made since Boveri first postulated that an abnormal karyotype could give rise to malignant cells. However, more research is needed to unravel mechanistic details of CIN mediated resistance, so that new and more effective drugs will be developed. Hopefully, this will have profound impact in the management of cancer disease and prevention of tumor relapse.

Conclusions

As conclusive part for this dissertation, I will resume here the main parts of this work. These are sort of "take home" messages to highlight the most important findings of this research.

- **Mad2 over-expression acts as tumor suppressive factor in breast tumorigenesis**

  The generation of new mouse models of breast cancer that recapitulated both oncogene dependence and chromosome instability provided the base to study breast tumorigenesis in a physiologically relevant model. The combined over-expression of Mad2 and either the c-MYC or Her2 oncogene upon completion of mammary gland development highlighted the suppressive role of exogenous Mad2 in primary tumor latency. Therefore, from this study we can conclude that Mad2 over-expression is detrimental in breast tumor formation and this effect is tissue dependent.

- **Mad2 over-expression promotes aneuploidy and tumor heterogeneity**

  As previously published (Sotillo et al., 2007), Mad2 over-expression causes an over-activation of the mitotic checkpoint, generating chromosome gains/losses, anaphase bridges and different types of rearrangements.
  Over-expression of an activated form of the Her2 oncogene in mouse (Montagna et al., 2002) promotes a certain level of aneuploid in primary tumors. Therefore, we wanted to understand whether combined Mad2 over-expression promotes higher levels of aneuploidy. Indeed, TI-Her2/Mad2 are significantly more aneuploid and are more prone to missegregation errors. Mad2 effect increased whole chromosome missegregation but did not affect segmental CIN.
  Hence, Mad2 over-expression promotes higher genomic heterogeneity which is also reflected by tumor histopathology.

- **Mad2 induced CIN induces therapy resistance**
  Aneuploid human tumors have poor prognosis and require shorter timeframe to relapse. Also in our mouse models, when the Her2 or MYC oncogene are co-expressed with Mad2, the time required for tumor recurrence is significantly shorter than in mice bearing the oncogene alone.
  Chromosome instability is one of the mechanisms able to promote the selection of oncogene independent clones, that are refractory to targeted therapy treatment. This results confirms what has been previously published in a CIN mouse model of lung adenocarcinoma. Thus, a general role for Mad2 over-expression in promoting tumor relapse is highlighted.

- **Mad2 induced CIN promotes heterogenetity in the mechanisms of relapse**

  TI-Her2/Mad2 and TI-MYC/Mad2 mice relapse over a variety of different molecular mechanisms. One possible explanation is that Mad2 over-expression in primary tumors generated the heterogeneity necessary to opt for alternative choices to relapse. Therefore, CIN offers the luxury of choosing among different molecular mechanisms to achieve oncogene independence. This notion translated into targeted therapy resistance in human patients.

Conclusiones

Como conclusión del trabajo aquí presentado, paso a resumir los puntos cruciales del proyecto.

- **La sobreexpresión de Mad2 actúa como supresor tumoral en mama**

  La generación de un nuevo modelo murino de cáncer de mama nos ha permitido recapitular tanto la dependencia oncogénica como la inestabilidad cromosómica, estableciendo la base para el estudio de la tumorogenesis en mama en un modelo fisiológicamente relevante. La sobreexpresión de Mad2 en combinación con dos oncogenes diferentes, c-MYC y Her2, nos ha permitido demostrar el papel supresor de Mad2 durante el desarrollo de tumores primarios de mama. Podemos concluir, por tanto, que la sobreexpresión de Mad2 es prejudicial para el desarrollo de tumores primarios de mama.

- **La sobreexpresión de Mad2 actúa como promotor de aneuploidía y favorece la heterogeneidad en tumores de mama.**

  Como se publicó anteriormente (Sotillo et al., 2007), la sobreexpresión de Mad2 provoca una hiperactivación del punto de control mitótico (SAC), dando lugar a perdidas y ganancias de cromosomas, aberraciones en anafase y diversos tipos de anomalías cromosómicas estructurales. La sobreexpresión de la forma activada del oncogén Her2 da lugar a ciertos niveles de inestabilidad cromosó mica en tumores primarios (Montagna et al., 2002). Uno de nuestros objetivos, por tanto, ha sido analizar si la sobreexpresión combinada de Mad2 y dicho oncogén provoca un aumento en los niveles de aneuploidía. Hemos podido confirmar que los ratones TI-Her2/Mad2 presentan niveles significativamente mayores de aneuploidía y una mayor tendencia a errores en la segregación cromosómica. Mad2 influye específicamente los errores en la segregación de cromosomas completos pero no así en la CIN segmental. Podemos concluir que la sobreexpresión de Mad2 promueve un aumento en la heterogeneidad genómica dando lugar a alteraciones en la histopatología de los tumores.

- **La inestabilidad cromosómica causada por la sobreexpresión de Mad2 otorga resistencia a la terapia clínica**

  Los tumores humanos aneuploides presentan una peor prognosis y requieren de un corto periodo de tiempo para dar lugar a recidivas. Del mismo modo, en nuestro modelos animales donde los oncogenes Her2 o MYC fueron co-expresados junto con Mad2, el tiempo requerido para la aparición de la recidiva fue significativamente menor al compararlo con aquellos que únicamente sobreexpresaban el oncogén. La inestabilidad cromosómica es uno de los mecanismos capaces de promover la selección positiva de

clones que crecen independientemente de la presencia del oncogén y que son, de esta forma, resistentes a la terapia dirigida. Cabe destacar, por tanto, el papel de la sobreexpresión de Mad2 en el proceso de recidivas tumorales.

- **La inestabilidad cromosómica inducida por Mad2 promueve heterogeneidad en las recidivas tumorales.**

  Los modelos de ratón TI-Her2/Mad2 y TI-MYC/Mad2 sufren recidivas a través de diversos mecanismos moleculares. Una de las posibles razones es que la sobreexpresión de Mad2 en tumores primarios otorgue la heterogeneidad necesaria para optar a diferentes alternativas que permitan el desarrollo de recidivas. Por tanto, la inestabilidad proporcionaría un escenario perfecto para la selección de diversas vías que permitieran la selección de clones independientes de la expresión del oncogén.

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